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Coordination of Phosphatidylserine and Phosphatidylethanolamine Metabolism in Mammalian Cells

by

Scot Stone

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Abstract

In animal cells, phosphatidylserine (PtdSer) is synthesized by two distinct synthases on the endoplasmic reticulum by a base-exchange reaction in which the polar head-group of an existing phospholipid is replaced with L-serine. The cloning and expression of cDNAs encoding murine liver PtdSer synthase-1 (PSS1) and PtdSer synthase-2 (PSS2) is reported in this thesis. The deduced amino acid sequence for murine PSS1 is ~30% identical to that of PSS2. PSS1 and PSS2 cDNAs were stably expressed in M.9.1.1 cells which are mutant Chinese Hamster ovary (CHO-K1) cells defective in PSS1 activity, are ethanolamine auxotrophs, and have a reduced content of PtdSer and phosphatidylethanolamine (PtdEtn). M.9.1.1 cells expressing PSS1 had increased serine-, choline- and ethanolamine-exchange activities, did not require exogenous ethanolamine for cell growth and the PtdSer and PtdEtn levels were restored to normal when cultured in the absence of exogenous ethanolamine, implying that the cloned cDNA encoded PSS1. M.9.1.1 cells expressing PSS2 had increased serine-, and ethanolamine-exchange activities suggesting that the cloned cDNA encoded PSS2. The growth defect of M.9.1.1 cells was only partially corrected in M.9.1.1 cells expressing murine PSS2 and PtdSer levels were restored to normal, but PtdEtn levels only were restored to normal when these cells were cultured with 20 μM ethanolamine. M.9.1.1 cells expressing PSS1 incorporated 3-fold and 2-fold more [^{3}H]serine into PtdSer than M.9.1.1 cells and CHO-K1 cells, respectively. M.9.1.1 cells expressing PSS2 only incorporated twice as much label into PtdSer as in M.9.1.1 cells, and did not exceed that in CHO-K1 cells. PSS1 and PSS2 cDNAs were expressed in McArdle rat hepatoma (RH) 7777 cells. McArdle RH7777 cells expressing PSS1, but not PSS2, incorporated more [^{3}H]serine into PtdSer than control cells. The levels of PtdSer and PtdEtn in cells expressing either PSS1 or PSS2 were not increased. Excess PtdSer produced by the PSS1-transfected cells was rapidly decarboxylated to PtdEtn and the degradation of PtdSer, and/or PtdEtn derived from

PtdSer, was increased. Moreover, the CDP-ethanolamine pathway for PtdEtn biosynthesis was inhibited in McArdle RH7777 cells expressing murine PSS1, but not PSS2. These data suggest that (i) PtdSer synthesis by murine PSS1 and PSS2 are differentially regulated, (ii) cellular levels of PtdSer and PtdEtn are tightly controlled, and (ii) the metabolism of PtdSer and PtdEtn is coordinately regulated in order to maintain phospholipid homeostasis.

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Table of Contents

CHAPTER 1 - Introduction to Phosphatidylserine and Phosphatidylethanolamine Metabolism 1

1.1 Introduction to Phosphatidylserine 2

1.2 Roles of Phosphatidylserine in Cellular Function 3

1.2.1 Activation of protein kinase C 4

1.2.2 Apoptosis 5

1.2.3 Progression of the blood coagulation cascade 6

1.2.4 Other roles of phosphatidylserine 7

1.3 Biosynthesis of Phosphatidylserine 8

1.3.1 Phosphatidylserine can be synthesized by several mechanisms 8

1.3.2 The base-exchange reaction 10

1.3.3 Substrate specificity of the base-exchange reaction 13

1.3.4 Isolation of mutants defective in phosphatidylserine biosynthesis 15

1.3.5 Cloning and expression of the cDNAs encoding PSS1 and PSS2 17

1.3.6 Regulation of phosphatidylserine synthesis 20

1.4 Introduction to Phosphatidylethanolamine 22

1.4.1 Function of phosphatidylethanolamine 22

1.5 Biosynthesis of Phosphatidylethanolamine 23

1.5.1 Phosphatidylethanolamine synthesis by base exchange 24

1.5.2 Synthesis by reacylation of lyso-phosphatidylethanolamine 24

1.5.3 Phosphatidylethanolamine synthesis by decarboxylation of phosphatidylserine 25

1.5.3.1 PtdSer decarboxylase 26

1.5.4 Phosphatidylethanolamine synthesis via the CDP-ethanolamine pathway 27

1.5.4.1 Ethanolamine kinase 28

1.5.4.2 CTP:phosphoethanolamine cytidylyltransferase (ET) 29

1.5.4.3 CDP-ethanolamine:1,2-DAG ethanolaminephosphotranferse (EPT) 31

1.6 Relative contribution of the CDP-ethanolamine pathway and decarboxylation of PtdSer to PtdEtn synthesis 32

1.7 Thesis Objectives 34

CHAPTER 2 - Materials and Methods 42

2.1 Tissue Culture 43

2.2 Cloning and PCR 43

2.2.1 Isolation of cDNA from a mouse liver λgt11 cDNA library 43

2.2.2 Cloning the cDNA for PSS1 from mouse liver 44

2.2.3 Construction of a full-length PSS1 cDNA 46

2.2.4 Isolation of cDNA for PSS2 from mouse liver 46

2.3 Stable transfection of mammalian cells 47

2.4 Radiolabels 48

2.5 Northern blot analysis 49

2.6 Enzyme assays 50

2.6.1 PSS assay 50

2.6.2 Enzymes of the CDP-ethanolamine pathway 51

2.6.2.1 Ethanolamine kinase assay 51

2.6.2.2 ET assay 51

2.6.2.3 EPT assay 52

2.7 Radiolabeling phospholipids of cells in culture 52

2.8 Phospholipid analysis 54

2.9 DAG assay 55

2.10 Triacylglycerol assay 55

2.11 Growth measurements of M.9.1.1 cells 56

2.12 Expression of PSS1 as a TrpE fusion protein in *E. coli* 56

2.13 Production of anti-PSS1 antibody 58

2.13.1 Recombinant TrpE/PSS1 antigen 58

2.13.2 Peptides 58

2.14 Other methods 59

CHAPTER 3 - Results 60

3.1 Isolation of the cDNA for PSS1 from murine liver 61

3.2 Murine liver PSS1 can complement the PtdSer biosynthetic of M.9.1.1 cells 63

3.3 PtdSer and PtdEtn metabolism in McArdle RH7777 cells expressing murine liver PSS1 cDNA 65

3.4 PtdEtn metabolism via the CDP-ethanolamine pathway in McArdle RH7777 cells expressing murine liver PSS1 cDNA 68

3.5 Isolation of the cDNA for PSS2 from murine liver 72

3.6 Murine liver PSS2 complements the ethanolamine auxotrophy of M.9.1.1 cells 73

3.7 PtdSer production by murine PSS1 and PSS2 is differentially regulated 75

3.8 Is PtdEtn utilized by PSS2 derived from PtdSer or the CDP-ethanolamine pathway? 76

3.9 Murine PSS1 and PSS2 activities can be post-transcriptionally regulated 78

3.10 Expression of murine PSS2 in McArdle RH7777 cells 80

3.11 Expression of murine PSS2 in McArdle RH7777 cells does inhibit PtdEtn synthesis from CDP-ethanolamine 81

3.12 Expression of ET in McArdle RH7777 cells 82

3.12.1 Expression of human ET cDNA does not increase CDP-ethanolamine or PtdEtn synthesis in McArdle RH7777 cells 82

3.12.2 Expression of rat liver ET cDNA increases CDP-ethanolamine, but not PtdEtn synthesis in McArdle RH7777 cells 84

3.13 Tissue distribution of PSS1 and PSS2 in murine tissues 86

3.14 Subcellular localization of PSS1 and PSS2 in murine liver 88

CHAPTER 4 - Discussion and Concluding Remarks 139

4.1 Cloning and expression of murine liver PSS1 and PSS2 in M.9.1.1 cells 140

4.2 Regulation of PtdSer synthesis by PSS1 and PSS2 142

4.3 Post-transcriptional regulation of murine PSS1 and PSS2 146

4.4 Tissue distribution and subcellular localization of PSS1 and PSS2 in murine tissues 146

4.5 Coordinate regulation of PtdEtn synthesis 148

4.6 Concluding remarks 152

CHAPTER 5 - Appendix 1 - Supplementary Figures and Data 155

References 168

List of Tables

Table 1. The enzyme activities of the CDP-ethanolamine pathway are not reduced in Mc/PSS1 cells 106

List of Figures

Fig. 1.1. Synthesis of PtdSer in yeast and prokaryotes from CDP-diacylglycerol and L-serine 37

Fig. 1.2. Biosynthesis of PtdSer and PtdEtn 38

Fig. 1.3. Proposed role for PSS1 and PSS2 39

Fig. 1.4. Synthesis of PtdEtn via the CDP-ethanolamine pathway 40

Fig. 3.1. Cloning the cDNA for PSS1 from mouse liver 91

Fig. 3.2. Nucleotide and predicted amino acid sequences of mouse liver PSS1 cDNA 92

Fig. 3.3. Comparison of the predicted amino acid sequences of PSS1 from CHO-K1 cells, human myeloblastic cells and mouse liver 93

Fig. 3.4. Comparison of the predicted amino acid sequences of PSS1 from murine liver and PSS2 from CHO-K1 cells 94

Fig. 3.5. Base-exchange enzyme activities of wild type CHO-K1 cells, M.9.1.1 cells and control M.9.1.1 cells expressing mouse liver PSS1 95

Fig. 3.6. Growth of M.9.1.1 cells and M.9.1.1 cells expressing mouse liver PSS1 96

Fig. 3.7. PtdSer and PtdEtn content of control M.9.1.1 cells and M.9.1.1 cells expressing mouse liver PSS1 97

Fig. 3.8. Base-exchange activities of control McArdle RH7777 cells and McArdle RH7777 cells expressing mouse liver PSS1 (Mc/PSS1) 98

Fig. 3.9. Mc/PSS1 cells have an increased rate of incorporation of [^{3}H]serine into PtdSer and PtdEtn compared to control cells 99

Fig. 3.10. The phospholipid composition of Mc/PSS1 cells is not altered 100

Fig. 3.11. The rate of turnover/degradation of [^{3}H]serine-derived PtdSer and PtdEtn in control cells and Mc/PSS1 cells is increased 101

Fig. 3.12. [^{3}H]ethanolamine-derived metabolites of the CDP-ethanolamine 102

pathway accumulate in Mc/PSS1 cells

Fig. 3.13. Rate of incorporation of [^{3}H]ethanolamine into PtdEtn is lower in Mc/PSS1 cells 104

Fig. 3.14. Rate of degradation of [^{3}H]ethanolamine-derived PtdEtn is not increased in Mc/PSS1 cells 105

Fig. 3.15. Triacylglycerol, but not diacylglycerol, levels are reduced in Mc/PSS1 cells 107

Fig. 3.16. Cloning the cDNA for PSS2 from mouse liver by PCR 108

Fig. 3.17. Nucleotide and predicted amino acid sequences of murine liver PSS2 cDNA 110

Fig. 3.18. Comparison of the predicted amino acid sequences of PSS1 and -2 from mouse liver and PSS2 from CHO-K1 cells 111

Fig. 3.19. Base-exchange enzyme activities of control M.9.1.1 cells and M.9.1.1 cells expressing mouse liver PSS2 112

Fig. 3.20. Expression of PSS2 cDNA in M.9.1.1 cells complements the growth defect of this cell line 113

Fig. 3.21. Expression of PSS2 cDNA in M.9.1.1 cells restores the PtdSer, but not PtdEtn, content of M.9.1.1 cells 114

Fig. 3.22. Incorporation of [^{3}H]serine into PtdSer and PtdEtn of control M.9.1.1 cells and M.9.1.1 cells expressing PSS1 or PSS2 115

Fig. 3.23. The turnover rate of [^{3}H]ethanolamine-derived PtdEtn in M.9.1.1 cells expressing PSS2 is not altered 116

Fig. 3.24. Metabolism of [^{3}H]serine-derived PtdSer and PtdEtn in control M.9.1.1 cells and M.9.1.1 cells transfected with PSS2 cells 117

Fig. 3.25. Northern blot analysis of PSS1 and PSS2 mRNAs 118

Fig. 3.26. Base-exchange activities of control McArdle RH7777 cells and McArdle RH7777 cells expressing PSS2 (Mc/PSS2) 119

Fig. 3.27. McArdle RH7777 cells expressing PSS1, but not PSS2, have an increased rate of incorporation of [^{3}H]serine into PtdSer and PtdEtn compared to control McArdle RH7777 cells 120

Fig. 3.28. Expression of human PSS1 in McArdle RH7777 cells increases serine exchange activity and increases the incorporation of [^{3}H]serine into PtdSer compared to control cells 121

Fig. 3.29. PtdSer and PtdEtn content McArdle RH7777 cells expressing PSS2 is not altered, compared to control McArdle RH7777 cells 122

Fig. 3.30. McArdle RH7777 cells expressing murine PSS1, but not PSS2, have a reduced rate of incorporation of [^{3}H]ethanolamine into PtdEtn compared to control McArdle RH7777 cells 123

Fig. 3.31. Expression of human ET in McArdle RH7777 cells 124

Fig. 3.32. McArdle RH7777 cells expressing human ET do not contain more [^{3}H]CDP-ethanolamine than control McArdle RH7777 cells 125

Fig. 3.33. McArdle RH7777 cells expressing human ET do not incorporate more [^{3}H]ethanolamine into PtdEtn compared to control McArdle RH7777 cells 127

Fig. 3.34. The PtdSer and PtdEtn content of McArdle RH7777 cells expressing human ET is not altered compared to control McArdle RH7777 cells 128

Fig. 3.35. Incorporation of [^{3}H]serine into PtdSer and PtdEtn of McArdle RH7777 cells and McArdle RH7777 cells expressing human ET 129

Fig. 3.36. Comparison of the predicted amino acid sequences of human and rat liver ET 130

Fig. 3.37. Expression of rat liver ET in McArdle RH7777 cells 131

Fig. 3.38. McArdle RH7777 cells expressing rat liver ET have increased content of [^{3}H]CDP-ethanolamine, but not PtdEtn, compared to control McArdle RH7777cells 132

Fig. 3.39. Incorporation of [^{3}H]serine into PtdSer and PtdEtn of McArdle RH7777 cells and McArdle RH7777 cells expressing rat liver ET 133

Fig. 3.40. Distribution of PSS1 and PSS2 gene expression in murine tissues 134

Fig. 3.41. PSS base-exchange activities of mouse tissues 135

Fig. 3.42. PSS activity in MAM, but not ER, is inhibited by choline, while ethanolamine inhibits PSS activity in both MAM and ER 137

Fig. 3.43. Expression of TrpE/PSS1 fusion protein in *E.coli* 138

Fig. 5.1. Base-exchange enzyme activities of M.9.1.1 cells and control M.9.1.1 cells expressing mouse liver PSS1 157

Fig. 5.2. The rate of turnover/degradation of [^{3}H]serine-derived PtdSer and PtdEtn in control McArdle RH7777 cells and Mc/PSS1 cells is increased 158

Fig. 5.3. [^{3}H]ethanolamine-derived metabolites of the CDP-ethanolamine pathway accumulate in Mc/PSS1 cells 159

Fig. 5.4. Rate of degradation of [^{3}H]ethanolamine-derived PtdEtn is not increased in Mc/PSS1 cells 161

Fig. 5.5. ET activity is not reduced in Mc/PSS1 cells 162

Fig. 5.6. Expression of murine PSS2 cDNA in M.9.1.1 cells complements the growth defect of this cell line 163

Fig. 5.7. The turnover of [^{3}H]ethanolamine-derived PtdEtn in M.9.1.1 cells expressing murine PSS2 is not altered 164

Fig. 5.8. Metabolism of [^{3}H]serine-derived PtdSer and PtdEtn in control M.9.1.1 cells and M.9.1.1 cells transfected with murine PSS2 165

Fig. 5.9. Incorporation of [^{3}H]serine into PtdSer and PtdEtn of McArdle RH7777 cells and McArdle RH7777 cells expressing human ET 166

Fig. 5.10. McArdle RH7777 cells expressing rat liver ET have increased content of [^{3}H]CDP-ethanolamine, but not PtdEtn, compared to control McArdle RH7777 cells 167

Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BES	N,N-Bis[2-hydroxyethyl]-2-aminoethanesulfonic acid
Ca^{2+}	calcium
CDP	cytidine diphosphate
CDP-choline	cytidine diphosphocholine
CDP-DAG	cytidine diphosphodiacylglycerol
CDP-ethanolamine	cytidine diphosphoethanolamine
CDP-serine	cytidine diphosphoserine
CHO-K1	Chinese hamster ovary
CMP	cytidine monophosphate
CPT	cholinephosphotransferase
CT	CTP:phosphocholine cytidylyltransferase
CTP	cytidine triphosphate
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagles medium
EK	ethanolamine kinase
EPT	CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase
ER	endoplasmic reticulum
ET	CTP:phosphoethanolamine cytidylyltransferase

G418	geneticin
kDa	kilodalton
MAM	mitochondria-associated membrane
Mc/PSS1	McArdle rat hepatoma cells expressing PSS1
Mc/PSS2	McArdle rat hepatoma cells expressing PSS2
MOPS	[N-morpholino]propanesulfonic acid
PCR	polymerase chain reaction
PKC	protein kinase C
PSD	phosphatidylserine decarboxylase
PLD	phospholipase D
PSS	phosphatidylserine synthase
PSS1	phosphatidylserine synthase-1
PSS2	phosphatidylserine synthase-2
PtdCho	phosphatidylcholine
PtdEtn	phosphatidylethanolamine
PtdSer	phosphatidylserine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide electrophoresis
TAG	triacylglycerol
18:0	stearic acid
20:4	arachidonic acid
22:6	docosahexaenoic acid

CHAPTER 1

INTRODUCTION TO PHOSPHATIDYLSERINE AND PHOSPHATIDYLETHANOLAMINE METABOLISM

1.1 Introduction to Phosphatidylserine

All cells, from the simplest prokaryotes to the most complex eukaryotes, contain membranes which serve as barriers between aqueous compartments. Most membranes are comprised mainly of different classes of phospholipids, cholesterol and protein. The most abundant phospholipid in eukaryotic cells is phosphatidylcholine (PtdCho), making up approximately 50% of the phospholipid in a cell membrane [1]. Phosphatidylethanolamine (PtdEtn), the second most abundant phospholipid in eukaryotes, constitutes approximately 20% of cellular phospholipid [1]. However, PtdCho is virtually absent in most prokaryotes making PtdEtn the most abundant phospholipid in these organisms [2]. There are also several less abundant classes of phospholipids, including phosphatidylinositol, sphingolipids, and phosphatidylserine (PtdSer), each comprising approximately 5-10% of a cell's phospholipid content, depending on the tissue examined [1].

The primary focus of this thesis will be the aminophospholipid, PtdSer. PtdSer is a membrane component of all prokaryotic and eukaryotic cells. PtdSer was first identified in the brain by Folch (1941-2,1948), after it was separated from cephalin, which is a mixture of PtdSer, PtdEtn and inositol phospholipid [3-5]. Examination of the distribution of PtdSer in liver revealed that it is found in most organelles in trace amounts, with the lowest concentration occurring in mitochondrial membranes and lysosomes [6, 7]. Analysis of the fatty acid composition of PtdSer from rat liver indicated that it contains primarily three different fatty acids; stearic acid (18:0), arachidonic acid (20:4) and docosahexaenoic acid (22:6) [8, 9]. The most prevalent fatty acids of rat liver PtdSer

are 18:0 and 20:4, each accounting approximately 40% of the fatty acid content of PtdSer, while 22:6 accounts for 6%. However, in brain gray matter, PtdSer is enriched in 22:6, which accounts for almost 40% of the total fatty acids of PtdSer [10, 11]. Generally, the PtdSer pool in brain and liver is enriched in stearoyl-polyunsaturated molecular species, such as 18:0/20:4 and 18:0/22:6 [12]. In most animal tissues, such as heart, kidney, spleen, liver, erythrocytes and lung, PtdSer accounts for less than 10% of the total phospholipids [1]. The brain, however, is enriched in PtdSer, comprising up 15% of total cellular phospholipid [1]. In the brain, PtdSer enriched in 22:6, appears to be essential for optimal function of the nervous system. PtdSer has been suggested to modulate benzodiazepine receptors [13], increase synaptic efficiency [14] and can facilitate the release of neurotransmitters, such as acetylcholine, in aging rats [15]. In addition, PtdSer can enhance binding of opiates [16] and inhibit binding of glutamate [17] to synaptic membranes.

1.2 Roles of Phosphatidylserine in Cellular Function

Although PtdSer only accounts for a small fraction of cellular phospholipids, it cannot be dismissed as having merely a structural function. As is the case with most phospholipids, PtdSer has an active role in many critical cellular processes. Three of the best documented functions of PtdSer are outlined below.

1.2.1 Activation of protein kinase C

It has long been established that protein kinase C (PKC) plays a key role in signal transduction pathways and is involved in regulating metabolism, gene transcription and cell growth [18]. Its activity therefore must be tightly regulated. PKC, which is normally inactive in the cytoplasm, is capable of binding to PtdSer on the inner leaflet of the plasma membrane with a low affinity, even in inactivated cells [19]. This interaction by itself does not stimulate PKC. Extracellular signals transmitted into a cell, usually by binding/activation of receptors localized to the plasma membrane, lead to the production of second messengers, such as inositol 1,4,5-trisphosphate and diacylglycerol (DAG), which results in an increase of intracellular calcium levels, which is required for the activation of calcium-dependent PKC isoforms [20]. PKC is only capable of binding to calcium in the presence of PtdSer since calcium binding sites on PKC do not exist in the absence of PtdSer [21-23]. However, the elevation of intracellular calcium seems to enhance translocation of PKC to the plasma membrane. The final requirement for activation of PKC is its association with, and binding to, DAG. The interaction of PKC, PtdSer, DAG and activating concentrations of calcium induces a conformational change in PKC increasing catalytic activity. Of the 12 isoforms of PKC identified, all require PtdSer, but not necessarily calcium, as a cofactor. The recognition of PtdSer by PKC is stereospecific for the L-serine moiety of PtdSer [21]. It has been hypothesized that PKC has three contact points with the phospho-L-serine head group of PtdSer, those being the amino, carboxyl and phosphate groups [21].

1.2.2 Apoptosis

A unique feature of PtdSer, as well as PtdEtn, is that these two aminophospholipids are restricted primarily to the inner leaflet of the plasma membrane, unlike PtdCho and sphingomyelin, which are found mostly on the outer leaflet [24, 25]. Plasma membrane lipid asymmetry is maintained by an aminophospholipid translocase that specifically moves PtdSer and PtdEtn from the outer to the inner leaflet of the lipid bilayer [26, 27]. This process is energy-dependent and can be inhibited by elevation of the intracellular calcium concentration. During apoptosis, there is a decrease in the activity of the aminophospholipid translocase, presumably from the influx of calcium into the cell, which prevents the movement of PtdSer from the outer to inner leaflet of the plasma membrane [28, 29]. In addition, during apoptosis, a phospholipid scramblase is activated by increased intracellular calcium [28]. This enzyme non-specifically transports all phospholipids bi-directionally across the plasma membrane resulting in a net movement of PtdSer to the cell surface. The movement of PtdSer to the outer leaflet of the plasma membrane serves as a highly specific signal for the recognition and removal of apoptotic cells by macrophages via an unidentified PtdSer receptor present on their plasma membrane [30-32]. Uptake of apoptotic cells by macrophages can be inhibited by the addition of an excess of PtdSer-containing liposomes, which compete for a PtdSer receptor on the surface of macrophages [30]. This putative PtdSer receptor is specific for PtdSer since other phospholipids, such as PtdCho, PtdEtn, phosphatidylinositol, phosphatidic acid and phosphatidylglycerol were incapable of competing with apoptotic

cells for macrophage binding sites. Phosphatidyl-D-serine was also unable to compete with apoptotic cells for macrophage binding sites, suggesting macrophage recognition is stereospecific for phosphatidyl-L-serine [30]. Soluble derivatives of PtdSer (glycerophosphoryl-L-serine and phospho-L-serine) are also able to compete with apoptotic cells for macrophage binding sites [30], suggesting that the polar head group of PtdSer serves as the recognition signal.

1.2.3 Progression of the blood coagulation cascade

The process of blood coagulation is essential for the survival of many organisms. This phenomenon requires the activation of platelets when a blood vessel is damaged or ruptured in order to form a clot to minimize blood loss [33, 34]. Activated platelets are able to interact with a large group of coagulation factors which allows for enhanced coagulant activity at the surface of the platelet. PtdSer plays a key role in the progression of the blood coagulation cascade and blood clot formation. As previously mentioned, a general feature of most cells is that PtdSer is restricted to the inner leaflet of the plasma membrane, including platelets [24] [25]. When blood vessels are cut, platelets become activated, the intracellular concentration of calcium in platelets increases which inactivates the aminophospholipid translocase and stimulates the phospholipid scramblase [25]. The consequence of this is the movement of PtdSer to the outer leaflet of the plasma membrane.

PtdSer on the cell surface of platelets is absolutely required for the progression of the blood coagulation cascade [35]. Membranes enriched in PtdSer serve as a catalytic

surface where coagulation factors are able to interact, therefore increasing their effective concentration. PtdSer is involved in two reactions of the blood coagulation cascade [36]. The first reaction involves the conversion of factor X into an active serine proteinase [24]. This requires the interaction of factor IX, another serine proteinase, factor VIII (PtdSer binding cofactor), calcium and PtdSer at the cell surface of the platelet. This complex enhances the efficiency of activation of factor X by 100,000-fold. Once factor X is activated, it is capable of binding to factor V and forms a complex with calcium and PtdSer at the cell surface producing the prothrombinase complex [24]. The prothrombinase complex converts prothrombin to thrombin allowing the progression of the coagulation cascade and clot formation to occur [24]. The necessity for PtdSer involvement in the coagulation cascade is well illustrated in a rare inherited bleeding disorder, termed Scott syndrome [37, 38]. This disorder is characterized by reduced thrombin formation. However, this is not due to defective coagulation factors. Instead, there is reduced mobilization of PtdSer to the cell surface due to defective phospholipid scrambling in activated platelets. Therefore, there is no active catalytic surface (i.e. PtdSer) which can interact with the coagulation factors.

1.2.4 Other roles of phosphatidylserine

PtdSer has been shown to form complexes with caldesmon [39], an actin, myosin and calmodulin binding protein, which is believed to play in role in regulating the actin cytoskeleton [40]. Initially, caldesmon was shown to bind to artificial PtdSer liposomes which caused the displacement of calmodulin. More recent studies have demonstrated

that this protein was capable of binding PtdSer in naturally occurring membranes, such as ER, and this binding occurs at the C-terminus of caldesmon [41, 42]. These investigators hypothesize that this PtdSer-caldesmon interaction may play a role in the organization of cytoskeleton and membrane structure.

PtdSer has also been demonstrated to be capable of interacting with and modulating the activity of some ion pumps. The sarcoplasmic reticulum of skeletal muscle contains an Ca^{2+}-ATPase which is capable of binding to PtdSer [43]. This intracellular Ca^{2+}-ATPase is inhibited when it associates with PtdSer. Additionally, PtdSer liposomes have been shown to be able to inhibit the Na^+/K^+ ATPase of synaptosomal plasma membranes [44]. In contrast, PtdSer appears to be required for the activation of the Na^+/K^+ ATPase from kidney and may actually comprise part of the cation access channel [45]. However, the interaction of PtdSer with these ion pumps has only been demonstrated *in vitro*. The physiological significance of these interactions has yet to be determined.

1.3 Biosynthesis of Phosphatidylserine

1.3.1 Phosphatidylserine can be synthesized by several mechanisms

Several different pathways for PtdSer biosynthesis have been observed. However, only two of these pathways are well established and account for most, if not all, of a cell's requirement for PtdSer. In prokaryotes, yeast and certain plant species, PtdSer is synthesized from L-serine and cytidine diphosphate (CDP)-DAG (Fig. 1.1). This reaction was initially demonstrated in the soluble fraction of *Escherichia coli* (*E.*

coli) where CDP-DAG and L-serine combine to produce PtdSer releasing cytidine monophosphate (CMP) [46]. However, in most other organisms that utilize this pathway, such as *Saccharomyces cerevisiae* [47] and some plants [48], PtdSer synthesis is localized to cellular membranes.

In all animal and some plant cells, PtdSer synthesis occurs by a calcium-dependent base-exchange reaction at the endoplasmic reticulum (ER), where the polar headgroup of an existing donor phospholipid, such as PtdCho or PtdEtn, is removed and replaced by an L-serine residue (Fig. 1.2) [49]. It is this biosynthetic pathway for PtdSer which will be the focus of this thesis. It is generally accepted that in animal cells, PtdSer is not synthesized from CDP-DAG since L-serine does not function as an acceptor for CDP-DAG [50]. Regardless of the mechanism of PtdSer synthesis, either by base-exchange or from CDP-DAG, PtdSer can be transported to mitochondria and can be decarboxylated to generate PtdEtn.

A pathway for the remodeling of PtdSer has also been suggested which could possibly serve to enrich PtdSer in 20:4 or 22:6 by a deacylation/reacylation mechanism. Incubation of radiolabeled 1-acyl-*sn*-glycero-3-phosphoserine with rat liver homogenates or microsomes produced PtdSer species enriched in 20:4 at the *sn*2 position of the molecule, but contained very little 22:6 [51]. It was determined that 22:6 is a poor acyl donor, compared to 20:4, indicating that it is unlikely that the stereospecificity of PtdSer could be achieved by remodeling via acylation [51, 52]. However, the stereospecificity of PtdSer, with respect to 22:6 could be achieved by alternative means. The enzyme(s)

catalyzing the base-exchange reaction may be specific for pools of PtdCho and PtdEtn containing 20:4 or 22:6, producing PtdSer enriched in with these fatty acids [12].

A fourth mechanism for PtdSer synthesis was originally proposed by Hubscher *et al.* (1958,1959) and by Bygrave, (1968,1969) in rat liver mitochondria [53-56]. It was postulated that the incorporation of serine into PtdSer was dependent on magnesium and ATP and was stimulated by CMP. It was proposed that this process probably involved intermediates such as CDP-serine and glycerophosphoserine which could be acylated to produce PtdSer [57]. It was later determined that the occurrence of the ATP-dependent pathway in mitochondria was due to contamination of the membrane preparation with microsomes [58]. It also became apparent that the reaction did not proceed through a series of water soluble serine-containing intermediates [59]. Additionally, the ATP-dependent pathway was found to actually occur through the serine base-exchange reaction. The base-exchange reaction can be stimulated by exogenous ATP by activating the Ca^{2+}-ATPase present on the ER. Consequently, calcium accumulates within the lumen of the ER activating the calcium-dependent serine base-exchange enzyme. The role of CMP in this process has still not been elucidated.

1.3.2 The base-exchange reaction

In animal cells, PtdSer synthesis occurs by a base-exchange reaction at the ER, where the polar headgroup of an existing donor phospholipid, such as PtdCho or PtdEtn, is removed and replaced by a serine residue (Fig. 1.2) [49, 60, 61]. This reaction has a requirement for calcium [49] and proceeds in an energy-independent manner [62].

However, the dependence of the base-exchange reaction for calcium can be circumvented by the presence of ATP. Voelker (1993) has shown that PtdSer synthesis in disrupted cells can be stimulated by ATP [63]. Presumably, ATP stimulates the calcium-ATPase of the ER resulting in the sequestration of calcium into the lumen of the ER. Synthesis of PtdSer by base-exchange does not result in a net increase in phospholipid mass. Instead, existing amino alcohol-containing phospholipids are modified. Originally, it was thought that base-exchange could be accounted for by the reversal of the phospholipase D (PLD) reaction, since both of these reactions involve the removal of the polar head group from phospholipid substrates. However, this possibility was ruled out due to several lines of evidence. First, PLD requires the presence of fatty acids such as oleic acid to become activated [64] while base-exchange activity is actually inhibited by fatty acids [65]. Second, the base-exchange reaction only uses amino alcohols, such as serine, ethanolamine and choline as substrates, whereas PLD utilizes a wide variety of alcohols. Third, exogenously-added calcium stimulates base-exchange activity but has no effect on PLD. Partially purified base-exchange preparations do not have PLD activity [66] and partially purified PLD preparations cannot incorporate serine into phospholipids [67].

The base-exchange pathway appears to be ubiquitous in most animal tissues examined. *In vitro* base-exchange activity has been detected in rat and mouse liver [61, 62, 68, 69], canine and rat heart [70, 71], rat, rabbit, chicken and guinea pig brain [72-74], hamster lung [75] and human leukocytes [76]. Additionally, subcellular localization studies of rat liver revealed that the serine base-exchange activity resided at the ER [50,

77, 78], and was enriched in a mitochondrial-associated membrane (MAM) fraction, that has ER-like properties [77, 79].

The primary product of the base-exchange reaction is generally considered to be PtdSer, but both ethanolamine and choline can also be incorporated into their respective phospholipids by base-exchange, *in vitro* [80-89]. Although PtdCho and PtdEtn can be synthesized by base-exchange, *in vitro*, the contribution to the overall synthesis of these two phospholipids, in intact cells, is unclear. In 1973, Bjerve determined that the major biosynthetic pathway for PtdSer in rat liver occurred by base-exchange, while the synthesis of PtdCho and PtdEtn by this mechanism was quantitatively insignificant [61]. Other investigators have also not been able to demonstrate significant synthesis of PtdCho or PtdEtn by base-exchange. Nagley and Hallinan, (1968) showed that the radiolabeled choline moiety of PtdCho in microsomes could not be exchanged for free choline [90]. Also, Stein and Stein (1969), through the use of double labeling experiments, could not demonstrate choline exchange in rat liver slices [86]. Additionally, two different mutant Chinese hamster ovary (CHO-K1) cell lines with defective synthesis of PtdCho [91] and PtdEtn [92], via their respective *de novo* pathways, have been isolated. These mutant cells incorporate reduced amounts of free choline or ethanolamine into their respective phospholipids indicating that if PtdCho and PtdEtn are produced by base-exchange, this pathway contributes very little to overall production of these lipids. It has been estimated that at physiological concentrations of ethanolamine, the base-exchange reaction is capable of producing <10% of cellular PtdEtn. Interestingly, when the concentration of ethanolamine is increased 20-fold, 40% of cellular PtdEtn has been

reported to be produced by base-exchange [93]. This does not exclude the possibility that PtdEtn or even PtdCho can be produced by base-exchange in very small quantities for specific functions.

Topology studies initially indicated that the serine and ethanolamine exchange activities were localized to the luminal side of rat brain microsomes, while the choline exchange activity was located on the cytosolic side [94]. However, subsequent studies in rat liver microsomal fractions contradicted these findings, indicating that the serine exchange activity was instead localized to the cytosolic side of the microsomes [78, 95]. This finding seems to support the hypothesis that PtdSer, and most other lipids, are synthesized at the cytosolic surface of the ER.

1.3.3 Substrate specificity of the base-exchange reaction

It has been demonstrated by several investigators that both PtdCho and PtdEtn can be utilized as phosphatidyl donors for PtdSer synthesis. However, it was unclear whether or not PtdCho and PtdEtn could exchange with all three bases: choline, ethanolamine and serine. Bjerve (1973) showed through the use of competition experiments with rat liver microsomes that the polar head group of PtdCho could be displaced with any of ethanolamine, choline or serine [68]. In contrast, the ethanolamine moiety of PtdEtn could only be displaced by ethanolamine or serine, but not choline. Experiments performed in CHO-K1 lysates also demonstrated that exogenously added PtdCho and PtdEtn could both serve as substrates for the synthesis of PtdSer by base-exchange [96] in a concentration-dependent manner.

The next obvious question which arose was: are there several base-exchange enzymes, each specific for a different phospholipid or base, or is there a single enzyme capable of catalyzing base-exchange with different phospholipid substrates? This led to several attempts to purify the base-exchange enzyme(s) in which substrate specificities could be examined. However, initial attempts of purification were unsuccessful since solubilization of the membrane-associated base-exchange activity with detergents resulted in the inactivation of the enzyme. Saito and Kanfer (1973) reported the first successful solubilization of the base-exchange enzyme from rat brain microsomes using the zwitterionic detergent, Miranol H2M [97]. This solubilized base-exchange enzyme required exogenously added phospholipids and calcium for activity and had a pH optimum of 7.2. Both PtdCho and PtdEtn were used as substrates for PtdSer synthesis, although PtdEtn was a better phosphatidyl donor. Unfortunately, due to the instability of the enzyme in the presence of detergent, subsequent purification became impossible. However, modifications of the solubilization procedure using Miranol H2M with sodium cholate allowed solubilization of a base-exchange enzyme that retained its activity. Subsequently, this solubilized, active preparation was then used for purification of the base-exchange enzyme [98]. Initial purification of the base-exchange enzyme resulted in the separation of the serine and ethanolamine exchange activities away from the choline exchange activity [66, 98]. This suggested that two distinct base-exchange enzymes might exist and that each possessed different substrate specificities.

In 1985, a more complex scheme for the purification of the base-exchange enzyme from rat brain was devised, which involved five purification steps [99]. The final result

was the copurification of serine and ethanolamine exchange activities. Both enzyme activities were enriched 25-fold over the initial microsomal preparation, indicating that the serine and ethanolamine exchange activities resided on one protein. The base-exchange enzyme was purified to near homogeneity as indicated by single protein band with an apparent molecular mass of 100 kDa after analysis by SDS-PAGE. The purified enzyme was able to utilize PtdEtn, but not PtdCho, for serine exchange, producing PtdSer. The pH optimum was 7.0 for serine and ethanolamine exchange and the optimum calcium concentration for serine exchange was 8 mM. Unfortunately, no further information about the purified enzyme has been made available.

1.3.4 Isolation of mutants defective in phosphatidylserine biosynthesis

As previously mentioned, purification of the enzymes involved in PtdSer synthesis was a very difficult process. Additionally, the possible presence of more than one base-exchange enzyme complicated studies when trying to determine the substrate specificity of this enzyme. Therefore, much of the focus moved away from studies involving the brain and liver tissues to examining PtdSer metabolism of cells in culture. Of particular use were CHO-K1 cells, which can relatively easily be genetically manipulated. Kuge *et al.* (1985), isolated a CHO-K1 mutant that had reduced choline, serine and ethanolamine exchange activities, *in vitro*, which was accompanied by reduced PtdSer synthesis in intact cells [100]. In addition, the synthesis of PtdEtn was also depressed, but PtdCho synthesis was unaffected. The authors therefore suggested that the primary function of the base-exchange enzyme is to synthesize PtdSer. Two other mutant CHO-

K1 cell lines have been isolated that are defective in PtdSer biosynthesis. Voelker and Frazier (1986), and Kuge *et al.* (1986) have both generated mutants, termed M.9.1.1 and PSA-3, respectively, in which serine and ethanolamine exchange activities are reduced by ~50% and have almost no detectable choline exchange activity, compared to wildtype CHO-K1 cells [96, 101]. Consequently, these cells require the presence of either exogenous PtdSer, PtdEtn or Etn for growth. In addition, both the M.9.1.1 and PSA-3 mutants had decreased synthesis of PtdSer and of PtdEtn from PtdSer and correspondingly reduced levels of these two phospholipids.

Since in both the PSA-3 and M.9.1.1 mutant cell lines ~50% of the serine exchange activity remains compared to CHO-K1 cells, both groups conclude that two base-exchange enzymes exist. Additionally, Kuge *et al.* (1986), showed that serine exchange activity in cell lysates could be inhibited by competition with either choline or ethanolamine in wildtype CHO-K1 cells, but in the PSA-3 mutant, only ethanolamine could inhibit serine-exchange activity, whereas choline had no effect [101]. Voelker and Frazier (1986), showed that wildtype CHO-K1 cells could utilize both PtdEtn and PtdCho as substrates for serine exchange to produce PtdSer [96]. However, the M.9.1.1 mutant was only capable of utilizing PtdEtn as a substrate for PtdSer biosynthesis. Both findings suggest that PtdSer synthesis can occur by two different serine exchange enzymes. PSS1 is capable of utilizing both PtdEtn and PtdCho as phosphatidyl donors, for exchange with serine, choline and ethanolamine. The other exchange enzyme, PSS2, can only use PtdEtn as a substrate, for exchange with serine and ethanolamine, but not choline.

PSA-3 cells were further mutagenized to generate a secondary mutant which had almost no detectable serine, ethanolamine or choline exchange activity [102]. This new mutant, PSB-2, required exogenous PtdSer for growth, but could not grow when supplemented with PtdEtn, indicating that PtdSer is essential for growth. Presumably, PSB2 cells could not grow because both PSS1 and PSS2 have been knocked out and consequently could not synthesize PtdSer from either PtdCho or PtdEtn. This was confirmed by showing that intact PSA-3 cells could convert exogenous PtdEtn to PtdSer, but PSB-2 cells could not. Additionally, both the rate of PtdSer biosynthesis in PSB-2 cells and PtdSer mass were lower than that of the PSA-3 mutant.

1.3.5 Cloning and expression of the cDNAs encoding PSS1 and PSS2

Up until now, the synthesis of PtdSer has been catalyzed by an enzyme referred to as the serine base-exchange enzyme. In more recent times, the enzyme has been renamed with respect to its biological function and will be referred to as PtdSer synthase (PSS). The enzyme that uses PtdCho as a substrate has been designated PSS1 and the enzyme that uses PtdEtn has been designated PSS2. A hypothetical scheme has been proposed to explain the presence of two PSS enzymes (Fig. 1.3) [103]. PtdCho is first used as a substrate by PSS1 to produce PtdSer, which can be decarboxylated to produce PtdEtn. The serine-derived PtdEtn can then be converted to PtdSer by exchange with serine, releasing free ethanolamine. This reaction would be catalyzed by PSS2. It is presumed that in CHO-K1 cells, PSS1 and PSS2 account for all of the serine-exchange

activity, each comprising approximately 50% of the activity. It is still unclear whether other cell types or tissues possess the same distribution of PSS1 and PSS2 activity.

The understanding of PtdSer synthesis was greatly enhanced by the isolation of the cDNAs for both PSS1 and PSS2 from CHO-K1 cells. The cDNA encoding PSS1 was isolated by complementation of the PSA-3 mutant cell line [104]. The predicted amino acid sequence of CHO-K1 PSS1 predicts a protein with a molecular weight of 55,000 and with several possible membrane spanning regions. However, immunoblot analysis indicated PSS1 had an apparent molecular weight of 42,000 and was localized to microsomes and MAM, and was absent from the cytosol and mitochondria [105]. This finding is consistent with the localization of PSS activity [50, 79, 105]. It was concluded that PSS1 does not undergo post-translational proteolytic modifications, which would account for its apparently reduced size on SDS-PAGE. Instead, it is believed that the extreme hydrophobic nature of PSS1 affects its migration during SDS-PAGE [106, 107]. Neither the cDNA nor protein sequence of PSS1 from CHO-K1 cells exhibited any homology to the PtdSer synthases of yeast [108-110], *E. coli* [111], *Helicobacter pylori* [112] or *Bacillus subtilis* [113]. This is not surprising since these enzymes catalyze very different biochemical reactions. Transfection of the PSS1 cDNA into PSA-3 cells showed that PSS1 had serine, choline and ethanolamine exchange activities and resulted in an increase in the rate of PtdSer synthesis compared to that in PSA-3 cells. Additionally, PSS1 transfected cells no longer required exogenous PtdSer or PtdEtn for growth and contained a normal PtdSer and PtdEtn content [104].

The cDNA for PSS2 has also been isolated from CHO-K1 cells [114]. Initially a human expressed sequence tag that had 30% sequence identity to PSS1 was identified as PSS2 by DNA database search analysis. A cDNA fragment was amplified by PCR from a CHO-K1 cDNA library using primers based on the human homologue sequence. This PCR product was then used as a probe to screen the cDNA library and isolate the cDNA encoding a putative PSS2. Comparison of the deduced amino acid sequences of PSS1 and PSS2 revealed 32% amino acid sequence identity [114]. No information regarding the subcellular localization of PSS2 is available yet. The cDNA of PSS2 encodes a protein with a predicted molecular weight of 55,000 that is most likely localized to cellular membranes since it contains several putative transmembrane domains. Expression of PSS2 cDNA in the PSA-3 mutant showed that PSS2 only possessed serine and ethanolamine, but not choline, exchange activities and was capable of stimulating PtdSer biosynthesis [114]. Previously, it was thought that PSS1 used both PtdEtn and PtdCho, while PSS2 used only PtdEtn, as phosphatidyl donors. However, studies in intact cells showed that PSS1 only catalyzes serine exchange with PtdCho, while PSS2 only catalyzes serine exchange with PtdEtn to synthesize PtdSer [102].

The isolation of the cDNAs encoding PSS1 and PSS2 from CHO-K1 cells made it possible to determine the substrate specificities of these two enzymes. PSB-2 cells, which have almost no detectable PSS activity, were transfected with either PSS1 or PSS2. PSB-2 cells were only able to synthesize PtdSer from PtdEtn when they were expressing PSS2, and could not use PtdCho as a phosphatidyl donor [102]. In contrast, PSB-2 cells expressing PSS1 were only able to utilize PtdCho as a substrate for PtdSer, and not

PtdEtn. It is still unclear why PSS1 is able to use both PtdEtn or PtdCho as substrates, *in vitro* [102, 115], but only can use PtdCho for PtdSer biosynthesis in intact cells.

It is also interesting to note that CHO-K1 cells require only one of PSS1 or PSS2 in order to grow [102]. PSB-2 cells transfected with PSS1 were found to be capable of growth without any lipid supplementation. PSB-2 cells, which lack both PSS1 and PSS2 activity, transfected with PSS2 are also capable of growth, however, these cells must be supplemented with PtdEtn, presumably as a substrate to produce PtdSer. These observations suggest that PtdSer is absolutely essential for cell growth.

1.3.6 Regulation of phosphatidylserine synthesis

Although the pathway for PtdSer biosynthesis has been extensively studied, exactly how this process is regulated is not well understood. Nishijima *et al.* (1986) demonstrated that *de novo* synthesis of PtdSer in CHO-K1 cells was inhibited by adding exogenous PtdSer to the culture medium [116]. Exogenously-added PtdSer resulted in decreased incorporation of radiolabeled phosphate into PtdSer, whereas other exogenously added phospholipids had no effect. Even though the addition of exogenous PtdEtn had no effect on PtdSer or PtdEtn synthesis, it did result in a 2-fold increase in PtdEtn mass. Although the synthesis of PtdSer was reduced, the PtdSer content of these cells remained unaltered, suggesting that the exogenous PtdSer was taken up by the cells intact and was incorporated into cellular membranes. No reduction in PSS activity was initially observed in total cell lysates. However, in a subsequent study in which PSS activity was measured in cellular membranes, it was determined that exogenously added

PtdSer decreased PtdSer synthesis by inhibiting PSS activity [117]. Subsequently, a CHO-K1 mutant cell line, mutant 29, was isolated in which the rate of PtdSer synthesis was increased three-fold compared to the parental cell line and could not be inhibited by exogenous PtdSer [118]. The nature of this insensitivity to PtdSer was traced to a point mutation in the PSS1 gene, the conversion of the amino acid arginine at position 95 to a lysine [117]. The authors believe that arginine-95 is part of an amino acid motif found in both PSS1 and PSS2 that is involved in regulating PtdSer production by an end product inhibition mechanism.

Evidence to further support the hypothesis of end product inhibition to regulate PtdSer production came from studies involving PSA-3 cells, a CHO-K1 mutant with reduced PtdSer biosynthesis due to a deficiency in PSS1 activity [114]. PSA-3 cells were transfected with the cDNA for PSS1 or PSS2, resulting in an increase in *in vitro* PSS activity, 16-fold and 13-fold, respectively above that in control PSA-3 cells, and 6-fold higher than in wild-type CHO-K1 cells. These transfected cells were able to synthesize more PtdSer than PSA-3 cells, but for neither PSS1 nor PSS2 did the rate of PtdSer biosynthesis exceed that of wild-type CHO-K1 cells.

A study by Kanfer *et al.* (1988) suggested that PtdSer synthesis is regulated by phosphorylation/dephosphorylation [119]. Addition of alkaline phosphatase to rat brain microsomes resulted in a decrease in PSS activity. In contrast, PSS activity was stimulated by the incubation of rat brain microsomes with cyclic AMP-dependent protein kinase, while conditions which favored PKC activation had no effect on serine exchange activity. These results must be interpreted with caution since the differences in PSS

activity observed in these experiments were small, and were generally statistically insignificant. Other studies examining the role of PKC in PtdSer synthesis have proved to be inconsistent. For example, Kiss *et al.* (1987), demonstrated that phorbol esters inhibited PtdSer synthesis in both homogenates and intact cells in a human leukemia cell line, but this effect was probably PKC independent [120]. However, there was rapid phosphorylation of nuclear protein 1b, which might be related to gene expression involved in the regulation of PtdSer synthesis.

As mentioned previously, calcium is essential for PSS activity at the ER [49], and therefore, the availability of calcium could represent a regulatory mechanism for PtdSer biosynthesis. PSS activity requires that calcium is sequestered within the lumen of the ER at mM concentrations, and that increases in cytosolic calcium concentrations actually inhibits PtdSer synthesis. Therefore, agents that modulate changes in intracellular calcium levels, such as glucose [121], glutamate [122], acetylcholine [122] and caffeine [123], can also affect PtdSer synthesis. Additionally, agents that inhibit the Ca^{2+}-ATPase of the ER, such as thapsigargin [123-125] and 2,5-di-tert-butylhydroquinone [63, 126], also inhibit PtdSer synthesis, since calcium levels in the lumen of the ER are depleted because calcium cannot be replenished.

1.4 Introduction to Phosphatidylethanolamine

1.4.1 Function of phosphatidylethanolamine

The role of PtdEtn has not been clearly defined yet this lipid appears to have very diverse functions. It obviously has a structural role due to its relative abundance. In

mammalian cells, it is the second most abundant phospholipid, next to PtdCho, comprising approximately 20% of cellular phospholipid [1]. In prokaryotes, which have virtually no PtdCho, PtdEtn is usually the most abundant phospholipid accounting for as much as 75% of cellular phospholipids [2].

PtdEtn has been shown to be the substrate for PtdCho biosynthesis in a unique pathway involving three subsequent methylations of PtdEtn [127, 128]. In mammalian cells, this pathway is only found in the liver, but the pathway is also present in yeast [129, 130].

PtdEtn is also involved in the inflammatory response. Plasmenylethanolamines, a class of PtdEtn, are enriched in 20:4 at the *sn*-2 position, which can be released from plasmenylethanolamines by the action of phospholipase A_2 [131-133]. The mobilization of arachidonic acid is essential for the production of prostaglandins and leukotrienes which can elicit a wide variety of biological responses. PtdEtn has also been implicated in the attachment of many cell surface proteins to the plasma membrane by glycosyl phosphatidylinositol anchors [134, 135]. The phosphoethanolamine moiety of the glycosyl phosphatidylinositol anchor linked to cell surface proteins is derived from PtdEtn [134-136].

1.5 Biosynthesis of Phosphatidylethanolamine

PtdEtn can be produced by several different pathways. The *de novo* synthesis of PtdEtn involves the incorporation of ethanolamine into PtdEtn via the CDP-ethanolamine pathway (Fig. 1.4) [129, 137]. PtdEtn is also made by exchange of phospholipid

headgroups with ethanolamine [49, 68], by decarboxylation of PtdSer (Fig. 1.2) [49] and by the deacylation/reacylation of PtdEtn [138]. Although PtdEtn can be generated by a number of different mechanisms, only the *de novo* pathway actually contributes to a net synthesis of phospholipid, while the other pathways all involve remodeling of existing phospholipids.

1.5.1 Phosphatidylethanolamine synthesis by base exchange

The base-exchange pathway has been discussed extensively in Section 1.3.2 and will not be repeated here. As previously mentioned, this pathway for PtdEtn synthesis contributes only a small portion (<10%) of PtdEtn produced and is considered to be quantitatively insignificant [139].

1.5.2 Synthesis by reacylation of lyso-phosphatidylethanolamine

A deacylation/reacylation mechanism has been described for PtdCho remodeling to generate phospholipids with defined molecular species and it was presumed that such a mechanism existed for PtdEtn remodeling as well [140-142]. However, initial studies by Samborski and Vance (1990), in which PtdEtn of isolated rat hepatocytes was radiolabeled with ethanolamine revealed that there was no significant acyl remodeling of PtdEtn [143]. Further investigation into PtdEtn remodeling revealed that PtdEtn derived from PtdSer, which contains predominantly 16:0 at the *sn*-1 position and 18:1 or 22:6 at the *sn*-2 position, was capable of undergoing deacylation/reacylation modification. The PtdEtn pool produced by this process was found to be enriched in 18:0/20:4, 16:0/20:4

and 16:0/18:1 molecular species [138]. Remodeling of PtdEtn by this mechanism would require the coordination of several enzymes, including phospholipases A_1 and A_2 and acyl-CoA:lysophospholipid transferases, and would require transport of these reaction intermediates between the mitochondria and the ER [138].

1.5.3 Phosphatidylethanolamine synthesis by decarboxylation of phosphatidylserine

PtdEtn can also be produced by the decarboxylation of PtdSer, a reaction catalyzed by the mitochondrial enzyme, PtdSer decarboxylase (PSD) (Fig. 1.2) [46, 49]. This pathway is of some importance since it has been shown that the bulk of mitochondrial PtdEtn is derived from the decarboxylation of PtdSer [144]. PtdEtn synthesis by this pathway is unique in that it occurs on the outer aspect of the inner mitochondrial membrane [145, 146]. The other pathways for PtdEtn biosynthesis are generally considered to be localized to the ER.

The transport of PtdSer from its site of synthesis, the ER, to the mitochondria, and transport of PtdSer across the outer mitochondrial membrane is required for the production of PtdEtn [147]. Experiments in permeabilized cells have shown that the transport of PtdSer to the mitochondria requires ATP and occurs in the absence of any cytosolic factors, which excludes the involvement of soluble phospholipid transport proteins and vesicular transport [63, 148, 149]. These results suggest a membrane bound transport mechanism. Voelker (1993) hypothesized that there is a physical connection between the ER and mitochondria where this transport process occurs [63]. Indeed, a membrane fraction has been isolated from rat liver that is closely associated with

mitochondria (MAM), but has ER-like properties [79]. It is believed that the MAM is associated with the outer mitochondrial membrane at sites which also contact the inner membrane [150, 151]. MAM is enriched in phospholipid biosynthetic enzymes, particularly PSS, and provides an excellent candidate for the channeling of PtdSer to the mitochondria [63, 77, 79]. It has also been shown that newly synthesized PtdSer is preferentially transported to mitochondria, possibly because "older" PtdSer has been integrated into the lipid bilayer of membranes and is not accessible for transport [65, 147]. Additionally, the rate of transport of newly-synthesized PtdSer from the ER to the mitochondria, and not the rate of PtdSer decarboxylation appears to limit the rate of PtdEtn production [152].

1.5.3.1 PtdSer decarboxylase

PSD catalyses the decarboxylation of PtdSer, generating PtdEtn. PSD is specific for PtdSer and does not decarboxylate serine, phosphoserine or glycerophosphoserine [153]. Most of the information available about PSD was obtained in studies from yeast. However, a cDNA encoding PSD has been isolated from CHO-K1 cells by complementation of the PSA-3 mutant, a cell line with defective PtdSer biosynthesis [101, 154]. Expression of the PSD cDNA in PSA-3 cells allowed these cells to grow without exogenous PtdSer and restored the PtdSer and PtdEtn levels to normal.

Genes encoding two distinct isoforms of PSD have been isolated from yeast and share 19% identity [155-157]. The PSD1 gene encodes a protein containing 500 amino acids that is localized to the mitochondrial inner membrane, and accounts for 95% of the

cellular PSD activity [155, 156]. Disruption of the PSD1 gene in yeast demonstrated that there was a small amount of PSD activity remaining, and that PSD1 activity was not essential for growth [156]. Presumably, sufficient PtdEtn can be produced by the CDP-ethanolamine pathway and PSD2. However, mitochondria from PSD1 deficient yeast have a dramatically reduced PtdEtn content and impaired mitochondrial function.

PSD2 accounts for the remaining 5% of cellular PSD activity of yeast and was surprisingly localized to the Golgi and vacuoles [157, 158]. The PSD2 gene is also not essential for growth since enough PtdEtn can be produced by PSD1 [157, 158]. The role of a non-mitochondrial PtdSer decarboxylase is not yet clear. Although PSD2 comprises only a small amount of total PSD activity, PSD2 is capable of producing enough PtdEtn for cell growth, even in the absence of the CDP-ethanolamine pathway. To date, only one form of PSD has been identified in mammals and is 43% identical to yeast PSD1. It is still unclear whether or not there is a PSD2 homologue in mammalian cells.

1.5.4 Phosphatidylethanolamine synthesis via the CDP-ethanolamine pathway

The *de novo* synthesis of PtdEtn via the CDP-ethanolamine pathway proceeds through a three step pathway involving several water soluble intermediates (Fig. 1.4) [129, 137]. Briefly, intracellular ethanolamine is first phosphorylated by ethanolamine kinase (EK) to phosphoethanolamine, which is converted to CDP-ethanolamine by CTP:phosphoethanolamine cytidylyltransferase (ET). Finally, CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EPT) transfers phosphoethanolamine from CDP-ethanolamine to DAG producing PtdEtn. This pathway is present in all

animal cells and yeast, but is absent from most bacteria, which generate PtdEtn by decarboxylation of PtdSer.

1.5.4.1 Ethanolamine kinase

The first committed step of the CDP-ethanolamine pathway is the phosphorylation of ethanolamine to phosphoethanolamine by EK. This reaction requires ATP and magnesium and is localized to the cytosol where EK exists as a soluble enzyme. Ethanolamine for this pathway can be supplied from a number of sources. For example, intracellular ethanolamine can be endogenously produced from PtdSer by base-exchange of PtdEtn with serine or choline. The intracellular degradation of PtdEtn by phospholipase D and the breakdown of sphingosine and sphinganine can also generate free ethanolamine [159, 160]. Additionally, ethanolamine can be readily taken up from the general circulation of animals obtained from the diet, since most animals have a serum ethanolamine concentration in the μM range [161].

It has been a point of controversy whether or not the same enzyme catalyses the phosphorylation of both choline and ethanolamine in mammalian tissues. Although the ethanolamine and choline kinase activities have been separated in lower eukaryotes and plants, purification from mammalian tissues indicated that both activities resided within the same protein [162-164]. Several cDNA clones for choline/ethanolamine kinase from various tissue have now been isolated [165-167]. The cDNA from rat liver was expressed in *E. coli* and was found to be able to phosphorylate both choline and ethanolamine providing further evidence that the activities reside in the same protein [167]. Purification

of choline/ethanolamine kinase from rat kidney revealed a possibly genetically distinct isoform since partial peptide sequences did not match any of the amino acid sequences from choline/ethanolamine kinase from human and rat liver [168].

1.5.4.2 CTP:phosphoethanolamine cytidylyltransferase (ET)

The second reaction of the CDP-ethanolamine involves the production of CDP-ethanolamine from phosphoethanolamine (Fig. 1.4). This reaction, which occurs in the cytosol, is catalyzed by ET, requires CTP, and is generally regarded as the rate-limiting step of the pathway. This was initially determined by incubating hepatocytes with increasing concentrations of ethanolamine which resulted in an increase in the incorporation of radiolabeled glycerol into PtdEtn [169]. The radiolabel also accumulated in phosphoethanolamine, but no increase in the CDP-ethanolamine pool, indicative of a rate-limiting step. Other evidence for ET being a rate-limiting enzyme was provided by studies by Tijburg *et al.* (1987). Incubation of hepatocytes with phorbol esters was found to stimulate PtdEtn synthesis, increase ET activity and reduce the size of the phosphoethanolamine pool [170].

Purification of ET from rat liver [171] allowed substrate specificity studies to be performed. CDP-ethanolamine and CDP-choline, a key intermediate in PtdCho biosynthesis produced by the enzyme, CTP:phosphocholine cytidylyltransferase (CT), were found to be produced by 2 different proteins: CT activity was absent from purified ET preparations [172]; ET activity, unlike CT activity, was not dependent on the

presence of lipids [173]; ET activity was localized to the cytosol, whereas active CT is associated with membranes [129].

The cDNA for ET was initially isolated from yeast by complementation of mutants defective in PtdEtn synthesis [174]. The predicted protein sequence of yeast ET had similarities to conserved catalytic regions of both yeast and rat CT. The first isolation of a mammalian ET cDNA was from a human glioblastoma cell line by complementation of a yeast mutant which had reduced ET activity [175]. Expression of the ET clone in this mutant showed that it encoded a protein with ET activity and was capable of producing CDP-ethanolamine. Northern blot analysis revealed that ET mRNA is present in several human tissues, with high levels in liver, heart and muscle. The cDNA for ET isolated from human glioblastoma cells was similar to other cytidylyltransferases, with the most similarity being to yeast ET (36%).

Analysis of the subcellular localization of ET revealed that unlike CT, ET activity is not regulated by reversible translocation to and from membranes [176]. ET is presumed to be a cytosolic protein that neither requires lipids for activity nor is tightly associated with membranes [173, 177, 178]. However, some association of ET with ER membranes has been detected by immunogold electron microscopy studies [173]. The cDNA sequences of ET [175] and CT [179] share some similarities, especially at the N-termini of the corresponding proteins. CT contains an amphipathic alpha helical C-terminal domain that has been proposed to mediate membrane association [180]. From analysis of the amino acid sequence of ET [175] it appears that ET might also have an amphipathic alpha helical domain close to the C-terminus that could potentially form a

loose association with membranes. It has been hypothesized that during homogenization of tissues ET that is loosely associated with membranes could become dislodged and appear to be a cytosolic enzyme. However, studies with permeabilized cells showed that ET activity leaked out of the cells at comparable rates to known cytosolic proteins, indicating that indeed the majority of ET likely resides in the cytosol [173].

1.5.4.3 CDP-ethanolamine:1,2-DAG ethanolaminephosphotransferase (EPT)

EPT catalyzes the final step of the CDP-ethanolamine pathway, transferring the phosphoethanolamine moiety from CDP-ethanolamine to 1,2-DAG, generating PtdEtn and CMP (Fig. 1.4). EPT is generally considered to be an integral membrane protein localized to the ER. However, Golgi has also been shown to possess EPT activity, which could not be accounted for by microsomal contamination [78]. EPT appears to be present in all eukaryotic cells, but is absent in prokaryotes, since they obtain their PtdEtn from decarboxylation of PtdSer [181].

The final step in PtdEtn biosynthesis involves the same biochemical reaction as for the synthesis of PtdCho. PtdCho is produced by the transfer of phosphocholine from CDP-choline to DAG, which is catalyzed by cholinephosphotransferase (CPT). EPT and CPT were shown to be separate proteins by studies of CHO-K1 mutants that had defective EPT activity but had normal CPT activity [182]. Additionally, yeast mutants have been isolated which have normal EPT activity, but defective CPT activity [183, 184]. EPT has never been purified to homogeneity due to inactivation by solubilization of the membrane bound enzyme with detergents. The gene encoding EPT has been cloned

from yeast by complementation of EPT-deficient yeast mutants [185]. The predicted amino acid sequence encodes a protein with a molecular weight of 45,000 with seven putative membrane spanning regions, consistent with its subcellular localization. Comparison of the EPT protein sequence to the yeast CPT gene product revealed that they share 54% similarity. A cDNA for EPT has not yet been isolated from a mammalian source.

EPT itself is not considered to be a regulatory enzyme in the CDP-ethanolamine pathway. However, there is evidence to suggest that the supply of DAG required for the production of PtdEtn from CDP-ethanolamine can be a limiting factor. It has been previously demonstrated that the supply of DAG can play a role in regulating PtdCho biosynthesis [186]. Incubation of hepatocytes with glucagon, cyclic AMP or okadaic acid resulted in reduced DAG levels with a subsequent decrease in PtdEtn synthesis [187, 188]. Tijburg *et al.* (1987) showed that treatment of hepatocytes with vasopressin stimulated PtdEtn production [189]. Although no significant change in EK or ET activities was observed after incubation of cells with vasopressin for 60 minutes, EPT activity was elevated slightly which was attributed to a 2-fold increase in DAG levels.

1.6 Relative contribution of the CDP-ethanolamine pathway and decarboxylation of PtdSer to PtdEtn synthesis

The presence of two major pathways for PtdEtn biosynthesis raises an important question: which pathway, if either, is responsible for the production of the majority of cellular PtdEtn? Generally, the CDP-ethanolamine pathway has been presumed to be the

predominant source of PtdEtn [190-192]. However, Voelker (1984) demonstrated that the decarboxylation of PtdSer accounted for 70-80% of cellular PtdEtn in baby hamster kidney cells, even when free ethanolamine was readily available for PtdEtn synthesis via the CDP-ethanolamine pathway [193]. Additionally, the production of CHO-K1 mutants with defective ET activity and consequently reduced PtdEtn synthesis via the CDP-ethanolamine pathway had a normal complement of PtdEtn [92]. The authors concluded that, in CHO-K1 cells, PtdSer serves as the major precursor for PtdEtn. Indeed, the general consensus is that the majority of PtdEtn in cells in culture is obtained from PtdSer.

If decarboxylation of PtdSer is capable of satisfying a cell's requirement for PtdEtn, the role of the CDP-ethanolamine pathway must be questioned. Some cell types, such as rat mammary carcinoma cells [194], hybridoma cells [195] and human keratinocytes [196] have been shown to require ethanolamine for growth and/or proliferation [197]. It has been suggested that the CDP-ethanolamine pathway produces a specific pool of PtdEtn, such as ethanolamine plasmalogen, required by these cells. In retinoblastoma cells and rat heart, kidney and liver, radiolabeled ethanolamine is readily incorporated into PtdEtn and ethanolamine plasmalogen [198, 199]. In contrast, very little ethanolamine plasmalogen is derived from the decarboxylation of PtdSer. PtdEtn synthesized from PtdSer may be utilized for other cellular functions such as glycosyl phosphatidylinositol anchors.

1.7 Thesis Objectives

In mammalian cells, PtdSer can be synthesized from base-exchange with either PtdCho or PtdEtn, a reaction catalyzed by PSS1 and PSS2, respectively. The reason why two different enzymes exist to produce PtdSer is not clear. Both enzymes generate PtdSer that is capable of being transported to mitochondria and is readily decarboxylated to PtdEtn.

Specific Aims:

1. It is probable that the sequences for PSS1 and PSS2 will be significantly conserved across species. Subsequently, the sequence information from the cDNAs for PSS1 and PSS2 isolated from CHO-K1 cells will be used to isolate the cDNAs encoding both PSS1 and PSS2 from murine liver. The isolation of cDNAs encoding PSS1 and PSS2 from murine liver will provide the tools necessary to study the properties of these enzymes by introducing them into cell culture model systems and examining their roles in phospholipid metabolism.
2. Both PSS1 and PSS2 from CHO-K1 cells have been shown to complement the PtdSer biosynthetic defect of the PSA-3 mutant. It will be determined if expression of murine liver PSS1 and PSS2 cDNA can eliminate the PtdSer biosynthetic defect of M.9.1.1 cells.
3. Most animal tissues appear to possess PSS activity, but the relative contribution of PSS1 and PSS2 to total tissue PSS activity has not been examined. The distribution of

PSS1 and PSS2 mRNA in murine tissues, such as testis, kidney, muscle, liver, lung, spleen, brain and heart, will be determined to assess the contribution of PSS1 and PSS2 to the net synthesis of PtdSer in these tissues.

4. ER and MAM both possess PSS activity. Additionally, PSS1 has been shown to reside in both microsomes and MAM, while the subcellular localization of PSS2 has yet to be elucidated. It is possible that PSS1 and PSS2 in murine liver each might be localized to the ER or MAM. Therefore, the subcellular localization of PSS1 and PSS2 in murine liver will be examined to determine whether or not these two enzymes reside in separate cellular compartments, or if they share a common localization.
5. Heterologous expression of these two cDNAs will allow the synthesis and metabolism of PtdSer to be examined and provide some insight into the cellular function of PSS1 and PSS2. The effects on phospholipid metabolism of expression of murine liver PSS1 and PSS2 cDNAs in McArdle RH7777 cells that have endogenous PSS activity will be observed. Additionally, PtdSer synthesis by PSS1 and PSS2 in CHO-K1 cells has been reported to be regulated by an end-product inhibition mechanism. It will be determined if PtdSer synthesis from murine liver PSS1 and PSS2 are also regulated by this mechanism.
6. PtdEtn metabolism has been demonstrated to be closely associated with PtdSer synthesis. Although it has long been known that PtdEtn can be produced by both the CDP-ethanolamine pathway and by decarboxylation of PtdSer, it has never been investigated in great detail whether or not these two pathways are capable of interacting to maintain PtdEtn homeostasis. There is some evidence to suggest that

the synthesis of PtdEtn from the CDP-ethanolamine pathway can be modulated by the production of PtdEtn from PtdSer. The consequences of increased synthesis of PtdEtn-derived from PtdSer due to expression of PSS1 and PSS2 on PtdEtn synthesis via the CDP-ethanolamine pathway will be studied. The possibility of reciprocal regulation of PtdEtn synthesis by the CDP-ethanolamine pathway and by decarboxylation of PtdSer will also be examined.

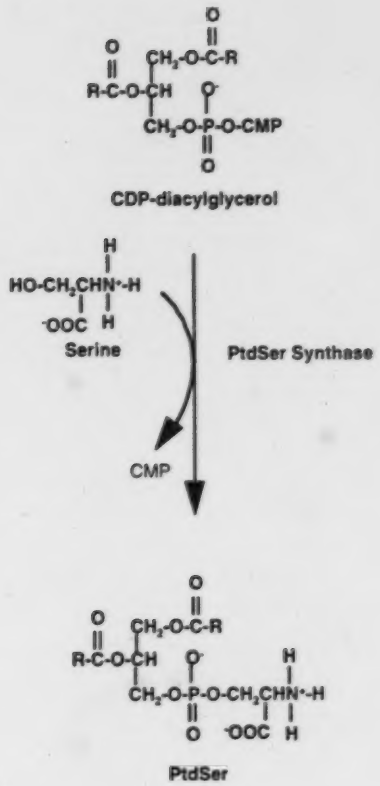


Fig. 1.1. Synthesis of PtdSer in yeast and bacteria from CDP-diacylglycerol and L-serine.

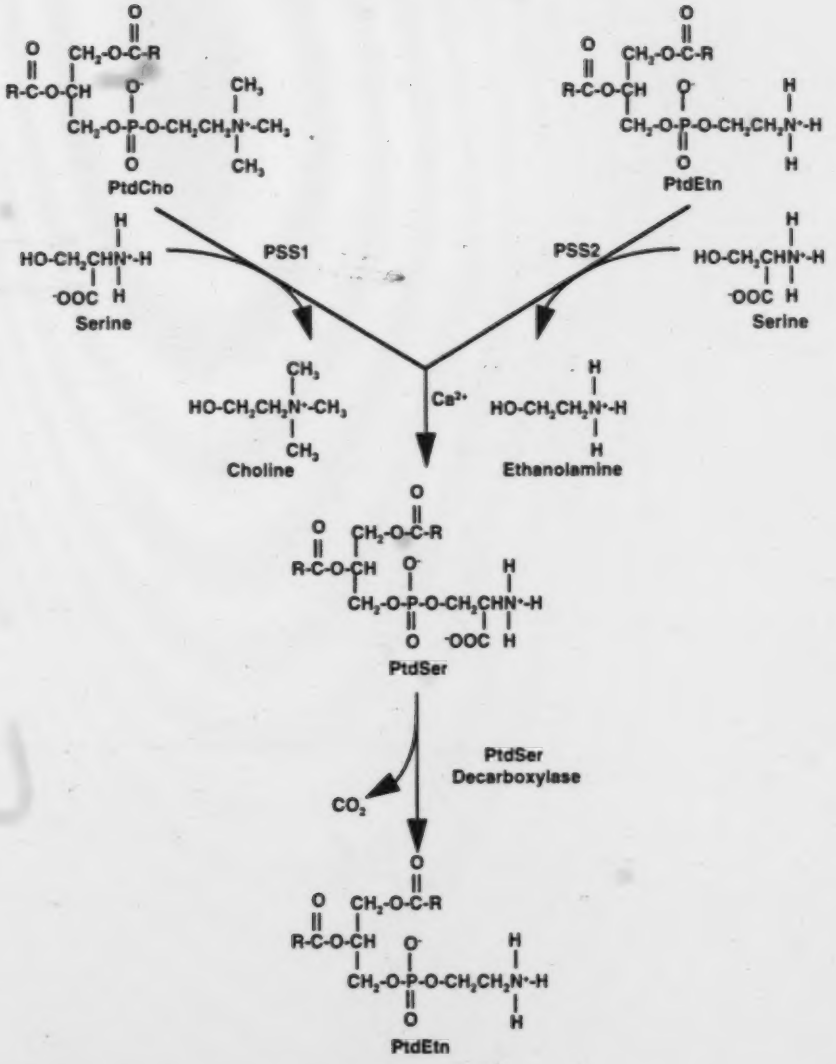


Fig. 1.2. Biosynthesis of PtdSer and PtdEtn

PtdSer is synthesized by removal of the polar head group of an existing phospholipid and replacing it with serine. This reaction is catalysed by the enzyme PtdSer synthase (PSS). PSS1 utilizes PtdCho as a substrate, while PSS2 uses PtdEtn. PtdEtn can be synthesized by the decarboxylation of PtdSer by the enzyme, PtdSer decarboxylase.

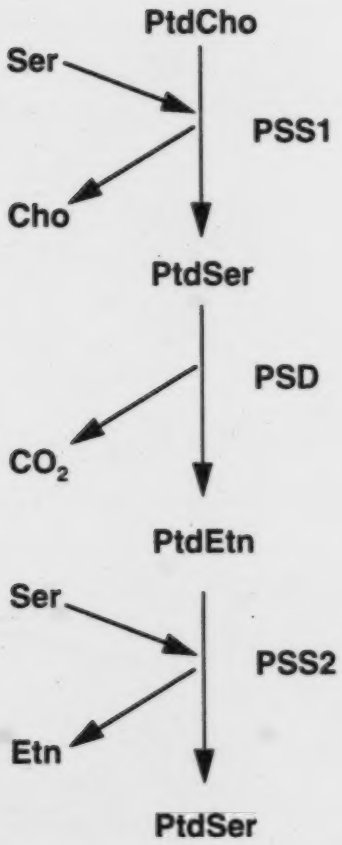
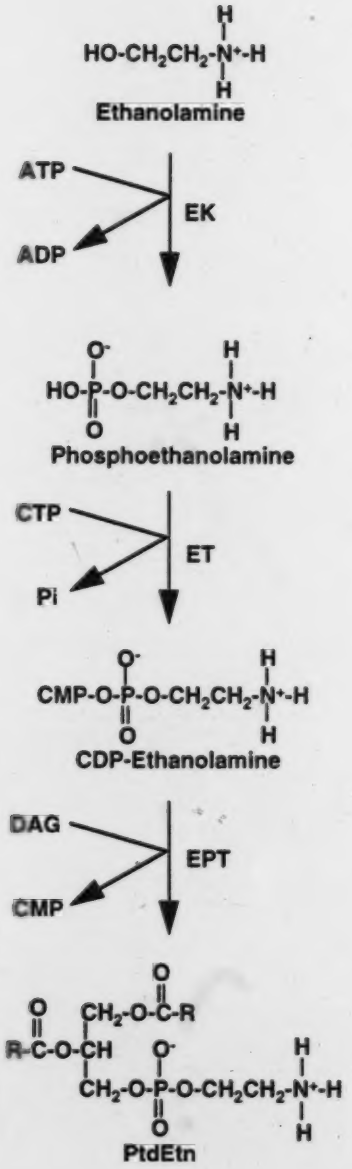


Fig. 1.3. Proposed role for PSS1 and PSS2

PSS1 first catalyses the conversion of PtdCho to PtdSer, which can then be decarboxylated to PtdEtn by PtdSer decarboxylase. Subsequently, PtdEtn-derived from PtdSer, can then be used by PSS2 to synthesized PtdSer, releasing free ethanolamine.

Fig. 1.4. Synthesis of PtdEtn via the CDP-ethanolamine pathway

Ethanolamine kinase (EK) phosphorylates ethanolamine to produce phosphoethanolamine. Phosphoethanolamine is then converted to CDP-ethanolamine by CTP:phosphoethanolamine cytidylyltransferase (ET). In the final step of the pathway, CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EPT) transfers phosphoethanolamine from CDP-ethanolamine to diacylglycerol to produce PtdEtn.


HO-CH2CH2-N+-H
Ethanolamine
ATP
ADP
EK
HO-P-O-CH2CH2-N+-H
Phosphoethanolamine
CTP
Pi
ET
CMP-O-P-O-CH2CH2-N+-H
CDP-Ethanolamine
DAG
CMP
EPT
PtdEtn

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Tissue Culture

CHO-K1 cells and McArdle RH7777 cells were obtained from the American Type Tissue Culture Collection and M.9.1.1 cells [96] were a gift from Dr. D.R. Voelker, National Jewish Research Center, Denver, CO. Fetal bovine serum (FBS), horse serum and tissue culture media were purchased from Gibco BRL. All cells were maintained at 37 ^{0}C with 5% CO_2. McArdle RH7777 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and 10% horse serum. CHO-K1 cells were cultured in Ham's F12 medium with 10% FBS, while M.9.1.1 cells were maintained in Ham's F12 medium with 20 μM ethanolamine and either 10% FBS or 10% delipidated FBS. Delipidated FBS was prepared by extracting FBS with diisopropyl ether and dialyzing in 0.9% NaCl [200].

2.2 Cloning and PCR

2.2.1 Isolation of cDNA from a mouse liver λgt11 cDNA library

All DNA modifying enzymes were purchased from Gibco BRL and New England Biolabs. The cDNA from a λgt11 mouse liver expression library (Clontech, Palo Alto, CA) was isolated by modification of the method described by Sambrook *et al.* (1989) [201]. Luria-Bertani medium (500 ml) was inoculated with Y1090R$^-$ strain *E. coli* infected with λ phage from the mouse liver cDNA library. Cells were grown at 37 ^{0}C until lysis was apparent after which addition of 10 ml of chloroform and further

incubation at 37 ^{0}C for 10 min completed lysis. DNAase I and RNAase A were added to the lysed cultures at final concentrations of 1 μg/ml and 5 μg/ml, respectively, and incubated at 37 ^{0}C for 30 min. Polyethyleneglycol and NaCl were added at final concentrations of 10% and 1 M, respectively. Cultures were maintained at 4 ^{0}C for 1 h to precipitate the phage, which were isolated by centrifugation for 10 min at 10,000 x *g*. The phage-containing pellet was resuspended in 10 mM Tris-HCl buffer (pH 8.0) containing 10 mM EDTA and 0.5% SDS, followed by incubation at 68 ^{0}C for 20 min to release the λ phage cDNA. The DNA solution was extracted three times with an equal volume of phenol:chloroform (1:1) and the DNA was precipitated by addition of NaCl (to a final concentration of 0.25 M) and 2 vol. 95% ethanol. The DNA was isolated by centrifugation for 10 min at 10,000 x *g*, washed with 80% ethanol and the DNA pellet was resuspended at a final concentration of 1 μg/μl in 10 mM Tris-HCl buffer (pH 8.0) containing 1mM EDTA, and used as the DNA template for PCR reactions.

2.2.2 Cloning the cDNA for PSS1 from mouse liver

Oligonucleotides were synthesized as primers for PCR. Modified λgt11 forward (FP) and reverse (FP) sequencing primers, and two gene-specific primers based on the CHO-K1 PSS1 cDNA sequence [115], were synthesized. The λgt11 sequencing primers were modified such that their length and GC content would be compatible with the PSS1 gene specific primers for PCR. The oligonucleotide 520S was complementary to the anti-sense strand of PSS1 and 913AS was complementary to the sense strand. (FP

= GCGACGACTCCTGGAGCCCG; RP = TGACACCAGACCAACTGGTAATG; 520S = GGCCATGAAGGCCTTGTTGATCCGTAGT; 913AS = TATGAATGTCCTTGAAGCTTGCCCA).

The PCR reaction mixture (50 µl) contained 25 ng of λ phage cDNA, 5 µl of the supplied 10-fold concentrated reaction buffer (Panvera, Madison, WI), 2.5 mM $MgCl_2$, 0.25 mM of each nucleotide triphosphate, 10 pmol of λgt11 sequencing primer (FP or RP) and 10 pmol of PSS1-specific primers (520S or 913AS), as well as 2.5 units of TaKaRa Ex Taq DNA polymerase (Panvera, Madison, WI). The PCR reaction was performed for 30 cycles at 94 ^{0}C for 1 min, 65 ^{0}C for 2 min, 72 ^{0}C for 1.5 min and a final 10 min extension at 72 ^{0}C. The products of the PCR reaction were separated by agarose gel electrophoresis and visualized by exposure to ultraviolet light after staining with ethidium bromide. The PCR reaction, containing the primers 913AS and FP, produced a 950 bp product corresponding to the 5' end of PSS1. The PCR reaction, containing the primers 520S and RP, produced a 1840 bp product corresponding to the 3' end of PSS1. These DNA fragments were extracted from an agarose gel slice using a gel extraction kit (Qiagen, Santa Clarita, CA) and each was cloned into the pCRII vector (Invitrogen, San Diego, CA). Inserts were sequenced by the DNA Core facility, at the University of Alberta, using an automated sequencer (Applied Biosystems Inc. Perkin-Elmer model 373A). The 950 bp 5' end and the 1840 bp 3' end were sequenced in both directions.

2.2.3 Construction of the expression vector containing murine liver PSS1 cDNA

The 950 bp 5' end that had been inserted into the PCRII vector was digested and ligated into the *KpnI/HindIII* sites of pBluescript (SK^+) (Stratagene). The 1840 bp 3' end that had been inserted into the PCRII vector was digested and ligated into the *ApaI/SpeI* sites of pBluescript (SK^+). The construct containing the 3' end was then digested with *HindIII/NotI*. The 3' end was ligated, in frame, into the vector containing the 5' fragment which had also been digested with *HindIII/NotI*. A 2 kb fragment containing the entire coding region of PSS1 was inserted into the eukaryotic expression vector pRc/CMV (Invitrogen).

2.2.4 Isolation of cDNA for PSS2 from mouse liver

Oligonucleotides were synthesized as primers for PCR. Two gene-specific primers were designed that were based on the CHO-K1 PSS2 cDNA sequence [114] and contained both the start and stop codons. The oligonucleotide 85ATGs was complementary to the anti-sense strand of PSS2 and 1516TAGas was complementary to the sense strand. (85ATGs = ATGCGGAGGGCCGAGCGCAGAGTC; 1516TAGas =ATCATGAGGCGGCTGAGGCCCCCT.)

The cDNA from a λgt11 mouse liver expression library (Clontech, Palo Alto, CA) was isolated by methods previously described in Section 2.2.1 and used as a template for PCR reactions. A 50 µl PCR reaction mixture contained 5 µl of the supplied 10-fold concentrated reaction buffer (Panvera, Madison, WI), 2.5 mM $MgCl_2$, 0.25 mM of each

nucleotide triphosphate, 200 ng of λ phage cDNA, 10 pmol each of 85ATGs 1516TAGas gene-specific primers, and 2.5 units of TaKaRa Ex Taq DNA polymerase. The PCR reaction was performed for 30 cycles at 94 ^{0}C for 1 min, 60 ^{0}C for 2 min, 72 ^{0}C for 1.5 min and a final 10 min extension at 72 ^{0}C. Agarose gel electrophoresis was used to separate the products of the PCR reaction. A 1.4 kb band was excised from the gel and the DNA fragment was extracted from an agarose gel slice using a gel extraction kit (Qiagen, Santa Clarita, CA) and cloned into the pCRII vector. Clones containing PSS2 cDNA were identified by Southern blotting using an internal oligonucleotide from PSS2 as a probe and positive inserts were sequenced in both directions. The cDNA encoding PSS2 was inserted into the eukaryotic expression vector pRc/CMV (Invitrogen).

2.3 Stable transfection of mammalian cells

Both M.9.1.1 and McArdle RH7777 cell lines were transfected by the calcium phosphate co-precipitation method [202]. Briefly, 10 μg of cDNA was added to 2x BES buffer (pH 6.95) to a volume of 600 μl. 400 μl of 0.315 M $CaCl_2$ was then added to the DNA solution dropwise while vortexing. The DNA-calcium suspension was incubated at room temperature for 30 min, vortexed for 60 sec and then added to a 100 mm dish containing cells at approximately 15% confluence and incubated overnight. Cells were shocked with 3 ml 15% glycerol in phosphate buffered saline (PBS) for 2 min followed by washing 2x with PBS. Fresh medium was added to the cells which were allowed to grow for 48 h. Cells were then split 1:2 and cultured in growth medium containing 600 μg/ml G418. Medium was routinely changed every 3-4 days. Once

individual colonies were formed and isolated, and cell lines established, the concentration of G418 was reduced to 200 μg/ml. Separate cultures of M.9.1.1 or McArdle RH7777 cells were also transfected with the expression vector containing no insert which served as controls.

The cDNA for human glioblastoma ET was a gift from Dr. J. Nikawa (Kyushu Institute of Technology, Japan). Human ET cDNA, containing the coding region, was inserted into the pRc/CMV expression vector (Invitrogen). The cDNA for rat liver ET was a gift from Dr. M. Houweling (University of Utrecht, the Netherlands) and was inserted into the pcDNA3.1 expression vector (Invitrogen). McArdle RH7777 cells stably expressing both human and rat ET were transfected by the calcium phosphate co-precipitation method described above.

2.4 Radiolabels

The radiochemicals [3-^{3}H]serine, [1-^{3}H]ethanolamine, [*methyl*-^{3}H]choline and [^{32}P]dATP were from Amersham (Oakville, ON). Cytidine-5'-diphospho-[1,2-^{14}C]ethanolamine was from ICN Radiochemicals (Montreal, Quebec). [1-^{3}H]phosphoethanolamine was prepared by the phosphorylation of [1-^{3}H]ethanolamine by purified choline kinase (Sigma). A reaction mixture containing 35 μCi of [1-^{3}H]ethanolamine, 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM ATP (pH 7.0) and 0.5 units of choline kinase was incubated at 37 ^{0}C for 1 h. The reaction was terminated by boiling for 5 min. Radioactive phosphoethanolamine was purified by thin-layer chromatography on silica gel G60 plates, in the solvent system, methanol:0.6%

NaCl:NH_4OH (10:10:1, v/v). Phosphoethanolamine was scraped from the plate and eluted from the silica with water. The solution was evaporated to dryness and resuspended in water to obtain a specific activity of 7.5 µCi/µmol, 2 mM..

2.5 Northern blot analysis [201]

mRNA was isolated from cells in culture using a MicroPoly (A) Pure mRNA Isolation Kit (Ambion, Austin, TX). Briefly, cells from five-150 mm dishes were harvested and disrupted in the lysis buffer supplied. Once purified, 11.25 µl of mRNA (5 µg) was denatured by adding 5 µl 10x MOPs buffer, 8.75 µl 37% formaldehyde and 25 µl formamide and incubated at 55 ^{0}C for 15 min. mRNA was separated by electrophoresis on a 1.2% agarose gel containing 1.1% formaldehyde. All solutions were treated with diethylpyrocarbonate to eliminate RNAase contamination. The gel was electrophoresed at 80 volts for 4 h, then soaked in RNAase-free water to remove formaldehyde. mRNAs were transferred to a Hybond-N membrane (Amersham Corp.) by capillary elution in 20 X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) overnight after which the membrane was allowed to dry for 4 h. Nucleic acids were fixed to the membrane by exposure to ultraviolet light for 5 min. The membrane was prehybridized in a solution containing 50% formamide, 6X SSC, 5X Denhardt's reagent, 100 µg/ml sheared salmon testes DNA, and 0.5% SDS overnight at 42 ^{0}C. A ^{32}P-labeled cDNA probe was then added to the prehybridization solution after it had been denatured by boiling for 10 min and then quick chilled on ice for 10 min. The membrane was hybridized overnight at 42 ^{0}C and then washes were performed at the following stringencies: PSS1, 1 x SSC at 60

^{0}C; PSS2, 0.1 x SSC at 60 ^{0}C; protein disulfide isomerase, 0.1 x SSC at 55 ^{0}C. ^{32}P-labeled PSS1, PSS2, protein disulfide isomerase and ß-actin cDNA probes were prepared using a random-primed DNA labeling kit (Gibco BRL) using 25 ng cDNA as a template and [^{32}P]dCTP. The cDNA for measurement of the abundance of protein disulfide isomerase mRNA was kindly provided by Dr. M. Michalak, University of Alberta.

2.6 Enzyme assays

2.6.1 PSS assay

PSS activity was measured as previously described [78]. Briefly, cells were scraped from dishes and disrupted by sonication with a probe sonicator, 2 x 10 s, in 10 mM HEPES buffer (pH 7.5) containing 0.25 M sucrose. Lysates were centrifuged for 30 min at 600 x *g* to pellet cellular debris and PSS activity was measured in the supernatant. Typically, 50-100 µg of protein was incubated with 25 mM HEPES buffer (pH 7.4), 4 mM hydroxylamine, 10 mM $CaCl_2$, and [3-^{3}H]serine (50 µCi/µmol, 0.4 mM), [1-^{3}H]ethanolamine (20 µCi/µmol, 0.2 mM), or [*methyl*-^{3}H]choline (50 µCi/µmol, 0.2 mM), in a final volume of 200 µl, for 20 min at 37 ^{0}C. The reaction was terminated by adding 5 ml of chloroform:methanol (2:1) and vortexing. 1.5 ml of water was added and the solution was vortexed and centrifuged at 3000 rpm on a bench top centrifuge. The upper aqueous phase was discarded and the lower organic phase was washed 3 x with 1.5 ml of methanol:water (1:1). The lower phase was evaporated to dryness under a stream of nitrogen gas, resuspended in 100 µl of chloroform and transferred to a scintillation vial and the radioactivity measured.

2.6.2 Enzymes of the CDP-ethanolamine pathway

Total cellular membranes and cytosol were prepared by centrifugation of cellular lysates at 400,000 x *g* for 30 min. Cytosol was assayed for EK and ET activities, membranes were assayed for EPT activity.

2.6.2.1 Ethanolamine kinase assay [203]

50-100 µg of cytosolic protein was incubated with 67 mM Tris-HCl (pH 8.5), 10 mM ATP, 11 mM $MgCl_2$ and 1.3 mM DTT and [1-^{3}H]ethanolamine (1 µCi/µmol, 1 mM) in a final volume of 100 µl, for 20 min at 37 ^{0}C. The reaction was stopped by boiling for 2 min. The products were separated by thin-layer chromatography on silica gel G60 plates, using the solvent system: methanol:0.6% NaCl:NH_4OH (10:10:1, v/v). The band corresponding to phosphoethanolamine was scraped and radioactivity determined.

2.6.2.2 ET assay [204]

50-100 µg of cytosolic protein was incubated with 10 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 5 mM DTT and [1-^{3}H]phosphoethanolamine (7.5 µCi/µmol, 0.2 mM) in a final volume of 100 µl for 20 min at 37 ^{0}C. The reaction was terminated by boiling for 2 min. The products were separated by thin-layer chromatography on silica gel G60 plates, using the solvent system: methanol:0.6% NaCl:NH_4OH (10:10:1, v/v). The band corresponding to CDP-ethanolamine was scraped and radioactivity determined.

In some experiments, as indicated, ET was assayed in lysates which had been prepared in the presence of the phosphoprotein phosphatase inhibitors microcystin (5 μM) [205], sodium orthovanadate (1 mM) and sodium fluoride (10 mM). Sodium orthovanadate was purchased from Calbiochem, La Jolla, CA. The phosphatase 1/2A inhibitor, microcystin, was a generous gift from Dr. C. Holmes, University of Alberta

2.6.2.3 EPT assay [78]

50-100 μg of total membrane protein was incubated with 50 mM Tris-HCl (pH 8.4), 1 mM EGTA, 1.6 mM 1,2-DAG, 10 mM $MgCl_2$ and cytidine-5'-diphospho-[1,2-^{14}C]ethanolamine (0.5 μCi/μmol, 0.4 mM) for 20 min at 37 ^{0}C. In some experiments, as indicated the DAG was omitted. The reaction was terminated by adding 5 ml of chloroform:methanol (2:1) and vortexing. 1.5 ml of water was added and the solution was vortexed and centrifuged at 3000 rpm on a bench top centrifuge. The upper aqueous phase was discarded and the lower organic phase was washed 3 x with 1.5 ml of methanol:water (1:1). The lower phase was evaporated to dryness under a stream of nitrogen gas, resuspended in 100 ml of chloroform and transferred to a scintillation vial and the radioactivity in PtdEtn was measured.

2.7 Radiolabeling phospholipids of cells in culture

Typically, McArdle RH7777 cells were split 1:20 and plated in 60 mm dishes with 3 ml growth medium. 24 h later, the medium was replaced with medium containing the desired radiolabel. M.9.1.1 cells were split 1:30, 48 h prior to the experiment, into 60

mm dishes with 3 ml Ham's F-12 medium containing 10% delipidated FBS and 20 μM ethanolamine. After 24 h, the medium was changed to Ham's F-12 with 10% delipidated FBS. 24 h later, the medium was replaced with the appropriate medium containing the desired radiolabel.

PtdSer and PtdEtn were radiolabeled by incubation of cultured cells with [3-^{3}H]serine (5 μCi/ml, 15 μCi/dish) in serine-free modified Eagle's medium. After the indicated time, the medium was removed and cells were washed 2x with PBS. Cells were scraped from the plate in 500 μl PBS, pelleted in a bench top centrifuge and resuspended in 200 μl PBS. A 10 μl aliquot was removed for protein determination and 50 μl was added to 5 ml chloroform:methanol (2:1) and vortexed. 1.5 ml of water was added and the solution was vortexed and centrifuged at 3000 rpm on a bench top centrifuge. The upper aqueous phase was discarded and the lower organic phase was washed 3 x with 1.5 ml of methanol:water (1:1). The lower phase was evaporated to dryness under a stream of nitrogen gas, resuspended in 50 μl of chloroform and applied to a silica gel G60 plate with authentic phospholipid standards (Avanti Polar Lipids, Birmingham AL). Phospholipids were separated by thin-layer chromatography using the solvent system: chloroform:methanol:acetic acid:formic acid:water (70:30:12:4:2, v/v). Phospholipids were visualized by exposure to iodine vapors. Radioactivity in the bands corresponding to PtdSer and PtdEtn was determined.

PtdEtn was radiolabeled by incubation of cultured cells with [1-^{3}H]ethanolamine (0.5 μCi/ml, 1.5 μCi/dish) in DMEM. Analysis of the incorporation of radiolabeled ethanolamine into PtdEtn was determined essentially as described above, except the

aqueous phase of the lipid extraction was saved for analysis of water-soluble intermediates of the CDP-ethanolamine pathway. Water-soluble intermediates of the CDP-ethanolamine pathway were separated by thin-layer chromatography on silica gel G 60 thin-layer plates in a combination of 2 different solvent systems. Ethanolamine and phosphoethanolamine were separated in the solvent system methanol:0.6% NaCl:NH_4OH, (10:10:1, v/v), whereas CDP-ethanolamine and glycerophosphoethanolamine were separated in the solvent system ethanol:0.9% NaCl:NH_4OH (80:10:26, v/v). Ethanolamine, phosphoethanolamine, CDP-ethanolamine and glycerophosphoethanolamine standards were added as carriers. The bands corresponding to these intermediates were scraped and radioactivity was measured.

2.8 Phospholipid analysis

Phospholipids were extracted from cells by the method of Bligh and Dyer [206] and separated by thin-layer chromatography on silica gel G 60 thin-layer chromatography plates in the solvent system chloroform:methanol:acetic acid:formic acid:water, 70:30:12:4:2 (v/v). Phospholipids were visualized by exposure to iodine vapors and identified by comparison with authentic standards. The phosphorus content of each phospholipid was determined [207]. Individual phospholipids were scraped from thin-layer chromatography plates into test tubes. Phospholipids were digested by heating at 180 ^{0}C for 30 min with 200 µl of 70% perchloric acid. Samples were cooled and 800 µl of water was added. The sample was vortexed and centrifuged at 3000 rpm in a bench top centrifuge to pellet the silica. 450 µl of the supernatant was transferred to a new tube and

2 ml of reaction solution was added and mixed well. The reaction solution contained 1% ammonium molybdate dissolved in 6 M HCl, 0.04% Triton X-100 and 0.15% malachite green oxalate (Sigma). The absorbance at 650 nm was read and lipid phosphorous was quantitated by comparison to a K_2HPO_4 standard curve (range= 2-20 nmol).

2.9 DAG assay

For measurement of the cellular content of DAG, lipids were separated by thin-layer chromatography using silica gel G60 plates in the solvent system heptane:isopropyl ether:acetic acid (60:40:4, v/v). The thin-layer plate was immersed in a solution of cupric acetate/phosphoric acid, then heated at 180 ^{0}C for 5 min to visualize the lipids [208]. The amount of DAG was determined by densitometric scanning of the bands and comparison with known amounts of standard DAG.

2.10 Triacylglycerol assay [209]

For measurement of the cellular content of TAG, lipids were separated as described in Section 2.9. The band corresponding to TAG was scraped from the thin-layer chromatography plate into a test tube. 1 ml of fresh hydroxylamine reagent was added to the sample and incubated at 65 ^{0}C for 2 min. Samples were cooled for 5 min and then 2.5 ml of fresh perchlorate reagent was added. After 30 min, the absorbance at 530 nm was read and TAG were quantitated by comparison to a triolein standard curve (range= 0-310 nmol). Hydroxylamine reagent was prepared by adding equal volumes of 4% ethanolic hydroxylamine and 8% ethanolic NaOH. After mixing, the solution was

centrifuged at low speed to remove precipitated NaCl. Stock ferric perchlorate was made by dissolving 5 g ferric perchlorate in 10 ml 70% perchloric acid, 10 ml of water and diluted to a final volume of 100 ml with cold absolute ethanol. The perchlorate reagent was made by adding 4 ml of stock ferric perchlorate with 3 ml 70% perchloric acid and diluted to 100 ml with 95% cold ethanol.

2.11 Growth measurements of M.9.1.1 cells [210]

Approximately 5 x 10^4 cells were plated in 60 mm dishes in Ham's F-12 medium containing 10% delipidated FBS, with or without 20 µM ethanolamine. At 24 h intervals, the medium was removed and cells were released from the dishes with 1 ml 0.25% trypsin in PBS for 2 min at 37 ^{0}C and harvested in 500 µl PBS. 100 µl of the cell suspension was added to 100 µl of Trypan blue and an aliquot was placed on a hemacytometer. Viable cells were counted by their ability to exclude Trypan blue.

2.12 Expression of PSS1 as a TrpE fusion protein in *E. coli* [211]

The N-terminal 50% of PSS1 was ligated, in frame, into the *SmaI/HindIII* sites of the pATH10 expression vector (Dr. A. Tzagoloff, Columbia University, New York). *E. coli* strain RR1 was transformed with either the pATH10 vector or the pATH10/PSS1 construct and were spread onto Luria-Bertani plates containing ampicillin (100 µg/ml) and 1.5% agar. A single colony was picked and used to inoculate 10 ml of fresh modified M9 media and allowed to grow at 37 ^{0}C with shaking until the OD_{600} was 0.3 (about 3 h). Modified M9 media contains 1x M9 salts (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl

and 0.1% NH_4Cl), 0.5% casamino acids, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% glucose, 10 µg/ml Thiamine hydrochloride (B1), 20 µg/ml L-tryptophan and 40 µg/ml ampicillin. The 10 ml culture was then diluted into 100 ml of modified M9 medium without tryptophan in a 2 l flask and allowed to grow at 37 ^{0}C for 2 h. Expression of the recombinant protein was induced by adding 500 µl of indoleacrylic acid (2 mg/ml) and the culture was incubated for another 4 h at 37 ^{0}C. Parallel cultures were also grown without induction by indoleacrylic acid.

After growth, bacteria were pelleted by centrifugation at 3000 rpm in a bench top centrifuge for 10 minutes. Cells were washed once in 20 ml 10 mM Tris-HCl (pH 7.5) and pelleted again. The cell pellet was resuspended in 20 ml 50 mM Tris-HCl (pH 7.5), 5 mM EDTA and 60 mg of lysozyme (Gibco BRL) and placed at 4 ^{0}C for 2 h. 1.4 ml of 5 M NaCl and 1.5 ml Nonidet P-40 were added and the solution was mixed by inversion. The viscous solution was sonicated with a probe sonicator (3 x 30 sec). Insoluble proteins were pelleted by centrifugation at 12,000 x *g* for 10 min at 4 ^{0}C. The insoluble proteins were washed once each with 20 ml 10 mM Tris-HCl (pH 7.5)/1 M NaCl and 10 mM Tris-HCl (pH 7.5). The insoluble proteins were resuspended in 1 ml 10 mM Tris-HCl (pH 7.5), which typically gave a protein concentration of 10 mg/ml. Proteins were then separated by SDS-PAGE and visualized by staining with Coomassie blue.

2.13 Production of anti-PSS1 antibody

2.13.1 Recombinant TrpE/PSS1 antigen

Approximately 1 mg of the insoluble protein fraction isolated from *E. coli* (Section 2.12) containing the recombinant TrpE/PSS1 fusion protein was separated by SDS-PAGE on a 12% polyacrylamide slab gel and stained with Coomassie blue. The band corresponding to the fusion protein was excised from the gel and cut into small pieces and frozen at -70 ^{0}C. The frozen gel was then lyophilized and ground into a fine powder with a mortar and pestle. One-fourth of the lyophilized powder was resuspended in 500 µl of PBS, emulsified with 500 µl of Freund's complete adjuvant and the solution was injected into a rabbit. Prior to injection, blood was drawn which was as used as a preimmune control. Four weeks later, the rabbit was inoculated with one third of the remaining lyophilized antigen which was emulsified with 500 µl of Freund's incomplete adjuvant. After two weeks, a blood sample was taken from the rabbit. The blood was allowed to clot, which was subsequently removed from the antiserum by low speed centrifugation. The antiserum was tested to determine if it recognized PSS1 by immunoblot analysis of mouse liver subcellular fractions and compared to immunoblots of the same fractions probed with preimmune serum.

2.13.2 Peptides

Two peptides based on the predicted amino acid sequence of the PSS1 cDNA from CHO-K1 cells were synthesized by the Alberta Peptide Institute. Peptides were linked to keyhole limpet hemocyanin to facilitate the production of an immune response.

Peptide 1 contained the sequence, AFTRDDSVPEDN, and corresponded to amino acids 57-68. Peptide 2 contained the sequence RHSKSKVTNGVGKK and corresponded to amino acids 458-471. 500 μg of each peptide was dissolved in PBS. Subsequent preparation and injection of the antigens into rabbits was as described in Section 2.13.1. Rabbits were boosted with antigen two and four weeks after initial injection. The antiserum was tested by immunoblot analysis of mouse liver subcellular fractions and compared to immunoblots of the same fractions probed with preimmune serum.

2.14 Other methods

All protein concentrations were determined by the BCA method (Pierce) using bovine serum albumin as a standard. MAM was isolated by the method of Vance [79]. ER was isolated as described by Croze and Morre' [212] as modified [78].

CHAPTER 3

RESULTS

A portion of this work has been published in *J. Biol. Chem* (1998) **273**, 7293-7302

3. RESULTS

3.1 Isolation of the cDNA for PSS1 from murine liver

The cDNA for PSS1 from murine liver was isolated by PCR cloning. The cDNA from a λgt11 cDNA library containing 2 x 10^6 clones was isolated and used as a template for PCR reactions. The 5' and 3' ends of PSS1 were amplified in two separate PCR reactions as described in Section 2.2.2, producing a 950 bp and 1840 bp fragment, respectively (Fig 3.1). The 5' and 3' fragments were ligated together at a common *HindIII* restriction site producing a complete PSS1 clone which was 2362 bp in length with the longest open reading frame encoding a 473 amino acid protein with a predicted molecular weight of 55,617 (Fig. 3.2) (GenBank accession # AF042731). The 5' untranslated region of the cDNA was 93 bp in length and contained an in-frame stop codon at position -45, suggesting that the PSS1 cDNA clone was full length. The 3' untranslated region was 943 bp in length and lacked the typical polyadenylation consensus sequence, AATAAA, but did however possess a poly A tail at the 3' end.

The activity of PSS has previously been shown to be localized to the ER and enriched in MAM [78]. Additionally, PSS1 protein is present in the microsomes and MAM of CHO cells as was determined by immunoblot analysis with an anti-PSS1 peptide antibody [105]. Although this data suggest PSS1 is specifically localized to the ER and MAM, it appears that the PSS1 protein lacks a typical N-terminal signal sequence for targeting the protein to the ER. However, the carboxyl terminus of PSS1 does contain a Lys-Lys motif which has been proposed to be an ER membrane retention signal [213]. PSS1 contains several putative membrane-spanning regions according to

hydropathy plot analysis suggesting that PSS1 is an integral membrane protein (Fig. 3.2) [214]. Many investigators have indeed demonstrated that PSS activity is tightly associated with membranes and can only be extracted by the addition of detergents [97].

Search analysis of GenBank with the murine PSS1 cDNA sequence detected a cDNA that has been isolated from a human myeloblast cell line and was deduced to encode a putative PSS1 (GenBank accession # D14694). Comparison of the cDNA sequences for PSS1 of mouse liver, CHO-K1 cells (GenBank accession #D90468) and human myeloblasts revealed that this enzyme is highly conserved across species, since their cDNA sequences are ~80% identical (Fig. 3.3) while the predicted amino acid sequences are >90% identical (Fig. 3.3). Most of the amino acid difference are conservative substitutions, which would not likely effect the properties of the enzyme. However, both the mouse liver and human myeloblast PSS1 amino acid sequences contain tyrosine and serine residues at positions 424 and 425, respectively, which are absent from the CHO-K1 sequence. The presence of a these potential phosphorylation sites may result in altered regulation of PSS1 activity compared to that of CHO-K1 cells. However, there is very little evidence to suggest that PSS1 activity can be regulated by phosphorylation. Kanfer *et al.* (1988) showed that incubation of rat brain microsomes with alkaline phosphatases inhibited serine, choline and ethanolamine exchange activity, whereas incubation with cAMP-dependent protein kinase stimulated all three exchange activities [119]. Experiments performed under conditions that favor phosphorylation by protein kinase C resulted in the stimulation of only choline exchange activity. However, any differences in base-exchange activity in these experiments were generally small and

not statistically significant and therefore one has to question whether there was any real effect.

Very little sequence homology was observed when the amino acid sequence of PSS1 from mouse liver was compared to the predicted amino acid sequence of PSS2, which has recently been cloned from CHO-K1 cells (Fig 3.4) [114]. Only 30% of the amino acids in mouse liver PSS1 are identical to those of the PSS2 cDNA, and most of this sequence homology occurs in two small clusters. PSS1 cDNA, from mouse liver, human or CHO-K1 cells do not share any homology to the PSS cDNAs isolated from *E. coli* [111], *S. cerevisiae* [108-110], *Helicobacter pylori* [112] or *B. subtilis* [113]. This lack of homology was not unexpected since the yeast and bacterial enzymes synthesize PtdSer by a different reaction from that in mammalian cells, and use CDP-DAG as a substrate instead of exchanging polar head groups of existing phospholipids.

3.2 Murine liver PSS1 can complement the PtdSer biosynthetic defect of M.9.1.1 cells

To confirm that the isolated mouse liver cDNA encoded PSS1, it was important to first determine whether or not the protein encoded by the PSS1 cDNA possessed PSS1 activity, and was capable of complementing the growth defect of M.9.1.1 cells and restoring their PtdSer and PtdEtn content to normal. Voelker and Frazier (1986) have reported that PSS activity in M.9.1.1 cells is 50% lower than in wild-type CHO-K1 cells, as indicated by a reduction in serine exchange activity [96], an observation that this data confirms (Fig. 3.5). It has been concluded that the defect in M.9.1.1 cells is in PSS1

activity, since a decrease in the choline and ethanolamine activities of M.9.1.1 cells was also observed, compared to wildtype CHO-K1 cells (Fig. 3.5). Stable expression of murine PSS1 cDNA in M.9.1.1 cells resulted in an approximately 5-fold increase in the incorporation of [3-^{3}H]serine into PtdSer in a cell-free assay compared to control M.9.1.1 cells transfected with a control vector (Fig. 3.5). In addition, PSS1 was capable of catalyzing base-exchange with both radiolabeled ethanolamine and choline as substrates. Compared to control M.9.1.1 cells which were transfected with the expression vector lacking an insert, a 4-fold increase in base-exchange activity for the incorporation of [^{3}H]ethanolamine into PtdEtn, and a 6-fold increase in the *in vitro* incorporation of [^{3}H]choline into PtdCho, were observed in M.9.1.1 cells expressing mouse liver PSS1 (Fig. 3.5). Since expression of the cloned mouse liver PSS1 cDNA increased the choline exchange activity of M.9.1.1 cells, in addition to the serine and ethanolamine exchange activities, the isolated cDNA must therefore encode PSS1, and not PSS2, which lacks choline exchange activity [114].

M.9.1.1 cells have been shown to be defective in the synthesis of PtdSer and, consequently, in the production of PtdEtn from PtdSer decarboxylation [96]. These cells require exogenously added PtdSer or ethanolamine (20 μM) in the culture medium in order to support sufficient PtdEtn synthesis via the CDP-ethanolamine pathway for normal growth (Fig. 1.2 and 1.4) [96]. Therefore, whether or not M.9.1.1 cells could be transformed into ethanolamine prototrophs by expression of mouse liver PSS1 cDNA was examined. As shown in Fig. 3.6, and as previously reported [96], M.9.1.1 cells grew in the absence of ethanolamine for approximately three generations, then died. However,

when these cells were cultured in medium containing 20 μM ethanolamine, normal growth was restored. M.9.1.1 cells expressing murine PSS1 were capable of normal growth both in the absence and presence of ethanolamine (Fig. 3.6). Thus, expression of murine PSS1 cDNA complemented the ethanolamine auxotrophy of M.9.1.1 cells.

Analysis of the phospholipid composition of M.9.1.1 cells revealed that PtdSer and PtdEtn levels were significantly [57% and 51%, respectively ($P< 0.05$)] lower when the cells were cultured in the absence of ethanolamine than in the presence of ethanolamine (Fig. 3.7), in agreement with previous data [96]. However, the content of PtdSer and PtdEtn in M.9.1.1 cells expressing mouse liver PSS1 cDNA, cultured in either the presence or absence of exogenous ethanolamine, was very similar to that of M.91.1 cells grown in the presence of 20 μM ethanolamine (Fig. 3.7). These results are consistent with those from studies by Kuge *et al.* (1991) in which expression of PSS1 cDNA from CHO-K1 cells corrected the growth defect and normalized the phospholipid composition of PSA-3 cells, which also have defective PSS1 activity [115].

3.3 PtdSer and PtdEtn metabolism in McArdle RH7777 cells expressing murine liver PSS1 cDNA

Murine PSS1 cDNA was expressed in McArdle RH7777 cells that express endogenous PSS1 activity. Several stably transfected McArdle RH7777 cell lines expressing mouse liver PSS1 were generated. One cell line, designated Mc/PSS1, was selected that exhibited a 2- to 3-fold increase in serine-, ethanolamine- and choline-exchange activities compared to control McArdle RH7777 cells (i.e. McArdle RH7777

cells transfected with the expression vector lacking an insert), when the cell lysate was assayed *in vitro* (Fig. 3.8). Although approximately 50 individual clones were screened, only 8 clones were isolated that possessed increased PSS1 activity, with a 3-fold increase in activity being the maximum. Possible explanations for this observation are that a higher expression of PSS1 might be lethal to the cells since they already possess endogenous PSS1 enzyme activity or PSS1 activity is tightly regulated and does not exceed a specific level of activation .

Mc/PSS1 cells and control cells were labeled with [3-^{3}H]serine for up to 6 h and incorporation of radiolabel into PtdSer and PtdEtn was determined. The total uptake of [^{3}H]serine was the same in the two cell types. In Mc/PSS1 cells, after 6 h of labeling, the amount of [^{3}H]serine incorporated into PtdSer was 3-fold higher than in control cells, and the amount of radiolabel incorporated into PtdEtn was double that of control cells (Fig. 3.9). These data indicate that the synthesis of both PtdSer and PtdEtn derived from PtdSer was increased in Mc/PSS1 cells compared to control cells. Since Mc/PSS1 cells have an increased rate of PtdSer and PtdEtn synthesis, it was logical to hypothesize that the phospholipid composition of these cells might be altered (i.e. the PtdSer and PtdEtn content might be increased). However, Fig. 3.10 shows that the amounts of the major phospholipids, except for sphingomyelin, were not significantly different in Mc/PSS1 cells and control cells. The reduction in sphingomyelin mass in Mc/PSS1 cells could be due to the increased synthesis of PtdSer causing the serine pool to be depleted. Serine is a substrate for sphingomyelin synthesis as well. The first committed step of sphingomyelin synthesis involves condensation of L-serine with palmitoyl-CoA

producing 3-ketosphinganine [215]. Therefore, an increased synthesis of PtdSer might leave less serine available for sphingomyelin synthesis.

These observations suggested that the levels of PtdSer and PtdEtn are tightly controlled and that synthesis and/or degradation of these lipids is a highly regulated process. Therefore, the metabolism of PtdSer and PtdEtn in control McArdle RH7777 cells and Mc/PSS1 cells was further examined. The cells were pulse-labeled for 1 h with [3-^{3}H]serine, then the radiolabel was chased for up to 12 h with 1 mM unlabeled serine (Fig. 3.11). As expected from the data shown in Fig. 3.9, incorporation of [^{3}H]serine into PtdSer in Mc/PSS1 cells at the end of the 1 h pulse was approximately 2.5-fold higher than in control cells (Fig. 3.11, upper panel). However, radioactivity in PtdSer declined more rapidly in Mc/PSS1 cells than in control cells during the chase period, indicating an increased rate of turnover or degradation of PtdSer in the cells expressing murine PSS1. Fig. 3.11 (lower panel) also shows that the incorporation of [^{3}H]serine into PtdEtn was consistently higher in Mc/PSS1 cells than in control cells, suggesting that at least a portion of the increased radiolabel lost from PtdSer in Mc/PSS1 cells was due to an increased conversion of PtdSer to PtdEtn. However, in neither Mc/PSS1 nor control cells was the radioactivity lost from PtdSer quantitatively recovered in PtdEtn. For example, in Mc/PSS1 cells during the first 6 h of the chase period, 107 x 10^3 dpm/mg protein were lost from PtdSer whereas the [^{3}H] content of PtdEtn increased by only 25 x 10^3 dpm/mg protein. Similarly, during the same time period, 35 x 10^3 dpm/mg protein were lost from PtdSer of control cells while only 15 x 10^3 dpm/mg protein accumulated in PtdEtn (Fig. 3.11). These data show that (i) in both types of cells some PtdSer was converted into

PtdEtn, (ii) the conversion of PtdSer to PtdEtn was increased in Mc/PSS1 cells compared to control cells, (iii) some radiolabeled PtdSer and possibly PtdEtn was degraded in both Mc/PSS1 and control cells, and (iv) in Mc/PSS1 cells, ~4 times as much radiolabeled PtdSer was degraded as in control cells. Presumably, these metabolic events are coordinated to ensure constant cellular levels of PtdSer and PtdEtn.

3.4 PtdEtn metabolism via the CDP-ethanolamine pathway in McArdle RH7777 cells expressing murine liver PSS1 cDNA

Figs. 3.9 and 3.11 show that Mc/PSS1 cells produced PtdEtn by the decarboxylation of PtdSer at an enhanced rate, compared to control McArdle RH7777 cells. However, the total cellular mass of PtdEtn did not increase indicating cellular phospholipids are maintained at constant levels (Fig. 3.10). Therefore, whether or not the increased expression of mouse liver PSS1 in McArdle RH7777 cells inhibited the synthesis of PtdEtn via the other major route for PtdEtn biosynthesis, the CDP-ethanolamine pathway, was examined. Mc/PSS1 cells were incubated with [1-^{3}H]ethanolamine for up to 1 h and the incorporation of radioactivity into the water-soluble intermediates of the CDP-ethanolamine pathway and PtdEtn were measured (Fig. 3.12 and 3.13). The total amount of [^{3}H]ethanolamine taken up by control McArdle RH7777 and Mc/PSS1 cells was the same. In Mc/PSS1 cells, more radiolabeled ethanolamine, phosphoethanolamine and CDP-ethanolamine was present than in control McArdle RH7777 cells (Fig. 3.12). In contrast, the incorporation of radiolabel into PtdEtn was greatly reduced in Mc/PSS1 cells compared to control McArdle RH7777 cells

(Fig. 3.13). The relative pool sizes of radiolabeled ethanolamine metabolites in Mc/PSS1 cells and control McArdle RH7777 cells were also compared after labeling with [^{3}H]ethanolamine for 24 h. As expected from the data shown in Fig. 3.12, ~13-fold more radiolabeled phosphoethanolamine was found to be present in Mc/PSS1 cells than in control McArdle RH7777 cells. In control McArdle RH7777 cells, the dpm/mg protein in phosphoethanolamine were $6.60 \pm 2.50 \times 10^3$ in control cells and $84.7 \pm 20.5 \times 10^3$ in Mc/PSS1 cells. For CDP-ethanolamine, $2.10 \pm 0.20 \times 10^3$ dpm/mg protein were recovered from control McArdle RH7777 cells and $3.40 \pm 1.40 \times 10^3$ dpm/mg protein from Mc/PSS1 cells.

Two likely explanations for the decreased labeling of PtdEtn from [^{3}H]ethanolamine when PSS1 was expressed are: (i) PtdEtn synthesis via the CDP-ethanolamine pathway was inhibited, or (ii) the rate of degradation of ethanolamine-derived PtdEtn was increased. These two possibilities were distinguished by incubation of Mc/PSS1 cells with [^{3}H]ethanolamine for 24 h. The radioactivity was chased with 2 mM unlabeled ethanolamine and the amount of [^{3}H]PtdEtn was measured. Fig. 3.14 shows that the rate of loss of radiolabel from PtdEtn was the same in Mc/PSS1 cells as in control cells. The combined data, therefore, indicate that the increased content of [^{3}H]ethanolamine-labeled intermediates in Mc/PSS1 cells, as shown in Fig. 3.12, was due to inhibition of PtdEtn synthesis via the CDP-ethanolamine pathway rather than increased degradation of ethanolamine-derived PtdEtn. Hasegawa *et al.* (1989), also demonstrated a similar finding in the CHO-K1 mutant, M29, that has increased PtdSer, and presumably PtdEtn, synthesis due to defective feedback regulation of PSS1 [118].

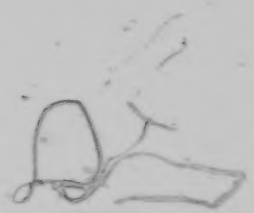
These M29 mutant cells incorporate less radiolabeled ethanolamine into PtdEtn, which accumulates in the phosphoethanolamine pool. Voelker and Frazier (1986), observed the opposite effect in the M.9.1.1 mutant [96]. In these cells, which have reduced PtdSer and PtdEtn synthesis, there was almost a 2-fold increase in the synthesis of PtdEtn by the CDP-ethanolamine pathway, presumably in an attempt regenerate cellular PtdEtn levels.

Since in Mc/PSS1 cells, less PtdEtn was synthesized by the CDP-ethanolamine pathway than in control McArdle RH7777 cells, it was possible that the activity of one of the enzymes of this pathway, most likely ET, was decreased in Mc/PSS1 cells. ET has been suggested to be the rate-limiting enzyme of the CDP-ethanolamine pathway under most metabolic conditions and its inhibition in Mc/PSS1 cells would account for the large increase in the size of the phosphoethanolamine pool [169, 170, 176]. However, Table I shows that the activities of ethanolamine kinase, ET and ethanolaminephosphotransferase in Mc/PSS1 cells were essentially the same as in control cells. Since many enzymes are regulated by phosphorylation-dephosphorylation events, ET was also assayed in cell lysates that had been prepared in the presence of the phosphoprotein phosphatase inhibitors microcystin [205], vanadate and fluoride, and the same results were obtained. However, there is currently very little evidence to suggest that ET is regulated by a phosphorylation/dephosphorylation cycle, so these results were not surprising. Tijburg *et al.* (1987), demonstrated that phorbol esters were capable of stimulating *de novo* PtdEtn synthesis by increasing ET and EPT activity slightly, but concluded these effects were not caused by direct phosphorylation of ET by protein kinase C [170].

The level of DAG has also been reported to regulate the CDP-ethanolamine pathway [189, 216] since this lipid is a substrate for ethanolaminephosphotransferase. The DAG content of control McArdle RH7777 cells and Mc/PSS1 cells was measured and it was found that the amount of this lipid was approximately the same in control cells (4.4 ± 0.5 nmol/mg protein) and Mc/PSS1 cells (4.0 ± 0.5 nmol/mg protein) (Fig 3.15). Since DAG is an intermediate in the biosynthesis of several important glycerolipids, and has also been implicated as a lipid second messenger, the cellular content of this lipid probably fluctuates only transiently and locally. EPT activity in Mc/PSS1 membranes, without adding exogenous DAG as a substrate for the assay, was measured (Table I). This method has been used to indirectly measure the DAG content of membrane fractions and might reveal small changes in DAG levels [217]. Although the specific activity of EPT was much lower when measured without exogenous DAG, there was no difference in EPT activity in control cells and Mc/PSS1 cells. In summary, changes in the supply of DAG for ethanolaminephosphotransferase were not detected by measurement of the total cellular or membrane DAG content. Since DAG is an intermediate for both phospholipid and TAG synthesis, we measured the cellular content of TAG as a potential "reserve" precursor pool of DAG. The content of TAG was 40 % less in Mc/PSS1 cells expressing PSS1 (14.2 ± 1.2 nmol/mg protein) than in control cells (23.7 ± 2.1 nmol/mg protein) (Fig 3.15). It is hypothesized that the increased capacity of Mc/PSS1 cells for phospholipid synthesis depletes the TAG pool, reducing the availability of glycerol-containing substrates for other phospholipid biosynthetic pathways.

3.5 Isolation of the cDNA for PSS2 from murine liver

A murine liver PSS2 cDNA (GenBank accession # AF099053) was isolated by PCR using a λgt11 cDNA library as a template and primers based on the sequence of PSS2 from CHO-K1 cells (GenBank accession # AB004109) [114]. The PCR reaction produced a 1.4 kb product, which was ligated into the pCRII vector (Invitrogen) and introduced into *E. coli* (Fig. 3.16A). 11 individual clones were screened for the presence of 1.4 kb inserts by restriction digestion with *HindIII*, 9 of which were positive (Fig. 3.16B). Southern blot analysis of these clones revealed that 7 of them contained an insert that hybridized to a PSS2-specific oligonucleotide probe (Fig. 3.16C). Clone #2 was sequenced which confirmed its identity as PSS2 (Fig 3.17). The cDNA sequence for PSS2 from mouse liver predicts a 473 amino acid polypeptide with a molecular weight of 55,000, and several potential membrane-spanning regions, as was also predicted for PSS2 for CHO-K1 cells [114] (Fig. 3.17). Alignment of the sequence of murine PSS2 with the cDNA and protein sequences of PSS2 from CHO-K1 cells revealed that the enzyme is highly conserved across species (Fig. 3.18). However, when the predicted amino acid sequence for PSS1 and PSS2 from mouse liver are compared, there is only ~30% identity and the sequences do not contain any long, continuous stretches of similarity (Fig. 3.18). These data strongly suggest that PSS1 and PSS2 from murine liver are encoded by different genes.



3.6 Murine liver PSS2 complements the ethanolamine auxotrophy of M.9.1.1 cells

As has been previously mentioned, M.9.1.1 cells are mutant CHO cells that are deficient in PSS1 activity, have reduced content of PtdSer and PtdEtn, and require one of ethanolamine, PtdSer, PtdEtn or lyso-PtdEtn for normal growth and survival [96]. Previously, the cDNA encoding murine PSS1 was stably expressed in M.9.1.1 cells which resulted in the elimination of the ethanolamine auxotrophy and restored a normal phospholipid composition [218]. Similar results were obtained when either PSS1 [115] or PSS2 [114] from CHO-K1 cells was expressed in another PSS1-deficient cell line, PSA-3. As confirmation that the cDNA isolated from murine liver encoded PSS2, the cDNA was expressed in M.9.1.1 cells. Since PSS1 and PSS2 share little homology and have different substrate specificities, it was important to determine whether or not the cDNA encoding PSS2 could also complement the growth defect of M.9.1.1 cells and restore their phospholipid composition.

It has been previously reported that the choline, serine and ethanolamine exchange activities of M.9.1.1 cells are 50% lower than that in wild-type CHO-K1 cells (Fig 3.19 inset) [96, 218]. Stable expression of murine PSS2 cDNA in M.9.1.1 cells resulted in an approximately 4-fold increase in serine exchange activity in cell lysates compared to control cells transfected with an empty expression vector (Fig. 3.19). Additionally, this increase in serine exchange activity was approximately 2-fold higher than that of CHO-K1 cells (Fig 3.19, inset). PSS2 was also able to utilize ethanolamine, but not choline, as substrates for base-exchange reactions. There was a 12-fold increase in base-exchange activity for the incorporation of [^{3}H]ethanolamine into PtdEtn, but no increase in the *in*

vitro incorporation of [^{3}H]choline into PtdCho in M.9.1.1 cells expressing mouse liver PSS2, compared to control M.9.1.1 cells (Fig. 3.19). These results confirm that the isolated cDNA encodes PSS2.

Voelker and Frazier (1986) have previously shown that M.9.1.1 cells require the culture medium be supplemented with ethanolamine, PtdSer, PtdEtn or lyso-PtdEtn in order to grow [96]. Exogenously added ethanolamine is presumably utilized to support PtdEtn synthesis via the CDP-ethanolamine pathway since the production of PtdEtn by decarboxylation of PtdSer is markedly reduced in these cells. The expression of murine PSS1 cDNA in M.9.1.1 cells has been demonstrated to bypass the growth requirement for ethanolamine [218]. Whether or not expression of murine PSS2 cDNA in these cells could also circumvent the mutant's requirement for exogenous ethanolamine was also examined. As shown in Fig. 3.20, M.9.1.1 cells grew for approximately three generations in the absence of ethanolamine and then died. However, these cells were capable of normal growth when 20 μM ethanolamine was included in the culture medium. M.9.1.1 cells expressing murine PSS2 cDNA were capable of growth both in the presence and absence of ethanolamine (Fig. 3.20). However, after 4 days, the growth rate of these cells was slightly slower than that of M.9.1.1 cells cultured in the presence of 20 μM ethanolamine. Despite this slower rate of growth, we conclude that expression of PSS2 cDNA is capable of complementing the ethanolamine auxotrophy of M.9.1.1 cells.

Mutant cell lines deficient in PSS1 activity (PSA-3 and M.9.1.1) have been demonstrated to have reduced PtdSer and PtdEtn levels [96, 101, 218]. However, the phospholipid composition of these cells are restored to normal by expression of the

cDNAs for PSS1. Since expression of murine PSS2 complemented the growth defect of M.9.1.1 cells, we wanted to determine if this also was capable of restoring the PtdSer and PtdEtn levels of these cells to normal. Fig 3.21 shows that when M.9.1.1 cells were cultured in the absence of ethanolamine, the PtdSer and PtdEtn content was significantly lower (40% and 41%, respectively) than of M.9.1.1 cells cultured in the presence of ethanolamine. In M.9.1.1 cells stably expressing murine PSS2 cDNA, the level of PtdSer was approximately the same, regardless of the presence or absence of ethanolamine in the culture medium, and was very similar to that of M.91.1 cells grown in the presence of 20 μM ethanolamine (Fig. 3.21). In contrast to PtdSer, the PtdEtn content recovered only when ethanolamine was present in the culture medium (Fig. 3.21). There was only a small increase in the level of PtdEtn when these transfected cells were cultured in the absence of ethanolamine. This is not consistent with the findings with PSS1 in which it was shown that M.9.1.1 cells expressing murine PSS1 had a normal PtdEtn content, even in the absence of ethanolamine [218]. These results suggest that since in intact cells PSS2 uses PtdEtn, but not PtdCho [102], as a substrate for PtdSer synthesis, ethanolamine supplementation is required to supply adequate amounts of PtdEtn from the CDP-ethanolamine for use by the increased activity of PSS2.

3.7 PtdSer production by murine PSS1 and PSS2 is differentially regulated

M.9.1.1 cells are defective in PSS1 activity and have reduced PtdSer and PtdEtn content (Fig. 3.7, 3.21). When the synthesis of PtdSer and PtdEtn in intact M.9.1.1 cells was examined, it was found to be reduced as well (Fig. 3.22). There was approximately a

44% and 50% reduction in the amount of [^{3}H]serine incorporated into PtdSer and PtdEtn in M.9.1.1 cells compared to wild-type CHO-K1 cells after labeling for 6 h. This is consistent with the ~50% reduction in serine exchange activity found in M.9.1.1 cells compared to CHO-K1 cells (Fig 3.19, inset). The incorporation of [^{3}H]serine into PtdSer and PtdEtn in M.9.1.1 cells expressing murine PSS1 or PSS2 cDNA was determined and compared to that of CHO-K1 cells and M.9.1.1 cells. In M.9.1.1 cells expressing murine PSS1, the amount of [^{3}H]serine incorporated into PtdSer and PtdEtn after a 6 h labeling period was 3-fold higher than control M.9.1.1 cells and 2-fold higher than wildtype-CHO-K1 cells (Fig. 3.22). In contrast, even though the *in vitro* serine exchange activity in M.9.1.1 cells expressing PSS2 was ~4-fold higher than in control M.9.1.1 cells and approximately twice as much label was incorporated into PtdSer and PtdEtn, as in control M.9.1.1 cells, the amount of [^{3}H]serine incorporated into PtdSer and PtdEtn after 6 h did not exceed that in CHO-K1 cells (Fig. 3.22). Since these cells had been deprived of ethanolamine 24 h prior to radiolabeling, it is possible that the ethanolamine pool was depleted, limiting PtdSer synthesis by PSS2 since PtdEtn is the primary substrate for this reaction. However, when cells were incubated with 100 μM ethanolamine 24 h prior to labeling or during the labeling period, the same result was observed.

3.8 Is PtdEtn utilized by PSS2 derived from PtdSer or the CDP-ethanolamine pathway?

Since PSS2 utilizes PtdEtn as its phospholipid substrate, we wanted to determine the source of PtdEtn used by this enzyme (i.e. CDP-ethanolamine pathway or PtdSer

decarboxylation). Therefore, the consumption of PtdEtn derived from the CDP-ethanolamine pathway in M.9.1.1 cells expressing murine PSS2 was examined. Control cells and M.9.1.1 cells expressing murine PSS2 were incubated with [^{3}H]ethanolamine for 6 h, after which the medium was removed and replaced with medium containing 2 mM unlabeled ethanolamine. The incorporation of label into PtdEtn was measured for up to 10 h. After the initial 6 h pulse with [^{3}H]ethanolamine, M.9.1.1 cells expressing murine PSS2 contained 44% less radiolabel in PtdEtn than did control cells (Fig. 3.23). This phenomenon had also been observed in McArdle rat hepatoma cells expressing murine PSS1 cDNA [218]. In Mc/PSS1 cells, which overproduce PtdEtn derived from PtdSer, it was demonstrated that PtdEtn synthesis from the CDP-ethanolamine pathway was inhibited in order to maintain cellular PtdEtn at a constant level. Alternatively, the reduced amount of radiolabel incorporated into PtdEtn in M.9.1.1 cells expressing murine PSS2 could indicate that PtdEtn derived via the CDP-ethanolamine pathway was being utilized for PtdSer biosynthesis by PSS2. However, there was very little difference in the rate of turnover of PtdEtn produced from ethanolamine during the 10 h chase period between control M.9.1.1 cells and M.9.1.1 cells expressing murine PSS2. Thus, PtdEtn required for PtdSer synthesis via PSS2 could come instead from PtdEtn derived from the decarboxylation of PtdSer. Alternatively, only a small fraction of the PtdEtn produced by the CDP-ethanolamine pathway may be utilized for PtdSer synthesis by PSS2, and its consumption might be too small to make a significant reduction in total PtdEtn mass and would not be detected in these labeling experiments. This hypothesis would be consistent with the data shown in Fig 3.21, where PtdEtn levels are decreased in M.9.1.1

cells expressing murine PSS2 grown without exogenous ethanolamine, compared to the same cells grown with ethanolamine. PtdEtn from the CDP-ethanolamine pathway could be consumed in order to generate PtdSer by PSS2, resulting in a decrease in PtdEtn levels, which cannot be regenerated since these cells have no source of ethanolamine.

In order to determine if PtdSer and PtdEtn metabolism in M.9.1.1 cells expressing murine PSS2 cDNA is similar to that observed in cells expressing murine liver PSS1 [218] we examined the turnover of PtdSer and PtdSer-derived PtdEtn. Cells were incubated with [^{3}H]serine for 6 h, after which the medium was replaced with medium containing 2 mM unlabeled serine. The incorporation of label into PtdSer and PtdEtn was then measured for up to 10 h. After the initial 6 h pulse, M.9.1.1 cells expressing PSS2 cDNA had incorporated approximately 1.5-fold more radiolabel into both PtdSer and PtdEtn compared to control cells (Fig 3.24), which was similar to what was observed in Fig 3.22. During the chase period, however, there appeared to be only a small increase in the rate of turnover of PtdSer and almost no change in the rate of PtdEtn labeling. If PtdEtn derived from PtdSer was the primary substrate for PSS2, then a more significant difference in PtdEtn labeling might be expected.

3.9 Murine PSS1 and PSS2 activities can be post-transcriptionally regulated

PSS1 and PSS2 mRNA levels were measured in CHO-K1 cells, control M.9.1.1 cells, and M.9.1.1 cells stably expressing murine PSS1 or PSS2 in order to determine if PSS activity in murine PSS1 and PSS2 transfected M.9.1.1 cells can be correlated with the level of expression of their corresponding mRNAs. Additionally, it is not yet known

whether the reduction in PtdSer synthesis in mutant M.9.1.1 cells is due to the expression of inactive PSS1, or if the PSS1 gene is not expressed. The mRNA content for PSS1 and PSS2 was assessed by hybridization with cDNA probes encoding murine PSS1, PSS2 and a loading control, protein disulfide isomerase (Fig 3.25, lower panel). The cDNAs encoding murine PSS1 and PSS2 used for transfection had been truncated in the 3'-untranslated region before insertion into the pRc/CMV expression vector. Consequently, the mRNAs are smaller than the endogenous PSS mRNAs. Fig 3.25 (upper panel) shows that there is less of the 2.4 kb PSS1 transcript present in control M.9.1.1 cells than in wild-type CHO-K1 cells, suggesting that the PtdSer biosynthetic defect in M.9.1.1 cells is due to reduced expression of the PSS1 gene, rather than normal expression of an inactive protein. In M.9.1.1 cells expressing murine PSS1, the amount of mRNA transcript representing murine PSS1 was ~14-fold higher than in control cells (Fig. 3.25, upper panel). However, the serine exchange activity in these cells was only 5-fold higher than in control cells [218]. Expression of the murine PSS2 mRNA in the four cell types is shown in Fig 3.25 (middle panel). The amount of endogenous PSS2 transcript (2.2 kb) appears to approximately the same in control M.9.1.1 cells, CHO-K1 and murine PSS1-expressing cells, suggesting that endogenous PSS2 gene expression was not significantly altered upon reduced PSS1 gene expression in M.9.1.1 cells or by expression of murine PSS1. However, the 1.4 kb murine PSS2 transcript was ~300-fold more abundant in M.9.1.1 cells stably transfected with murine PSS2 than in control cells. The 4-fold increase in serine exchange activity in these cells compared to control cells was not proportional to the 300-fold increase in the level of murine PSS2 mRNA. These

experiments suggest that the expression of murine PSS1 and, especially murine PSS2, might be regulated post-transcriptionally.

3.10 Expression of murine PSS2 in McArdle RH7777 cells

Several stably transfected McArdle RH7777 cell lines expressing murine PSS2 were generated. The cell line expressing the highest serine and ethanolamine-exchange activities in a cell free assay (2.5-fold and 5-fold, respectively) compared to control McArdle RH7777 cells was selected for subsequent experiments and was given the designation, Mc/PSS2 (Fig. 3.26). Although serine and ethanolamine exchange activities were elevated 2.5-fold and 5-fold, respectively, in Mc/PSS2 cells, as expected there was no increase in choline exchange activity (Fig. 3.26). Previously, it has been shown that McArdle RH7777 cell expressing murine PSS1 cDNA have an increased capacity for both PtdSer and PtdEtn synthesis [218]. The incorporation of [^{3}H]serine into PtdSer and PtdEtn was measured in Mc/PSS2 cells to determine if the increase in *in vitro* activity in these cells reflected an increase in the biosynthetic rate of these two phospholipids. The total uptake of [^{3}H]serine by Mc/PSS2 and control McArdle RH7777 cells was the same. The incorporation of [^{3}H]serine into PtdSer and PtdEtn after 6 h was unaltered by the expression of murine PSS2 (Fig. 3.27). This is in contrast to our previous findings with PSS1, in which McArdle RH7777 cells expressing murine PSS1 cDNA had 3-fold increased *in vitro* serine exchange activity and a proportional increase in the incorporation of [^{3}H]serine into PtdSer and PtdEtn compared to control McArdle RH7777 cells [218]; these data are included in Fig. 3.27 for comparison. Additionally, expression of PSS1

from human myeloblast cells (a gift from Dr. N. Nomura) in McArdle RH7777 cells also resulted in a ~2-fold increase in PSS activity compared to control McArdle RH7777 cells (Fig. 3.28, panel A) and was accompanied by an ~2-fold increase in the amount [^{3}H]serine incorporated into PtdSer (Fig. 3.28, panel B). Analysis of the PtdSer and PtdEtn content of Mc/PSS2 cells revealed no change in the PtdSer and PtdEtn levels of these cells compared to control McArdle RH7777 cells (Fig. 3.29). This result was not surprising since Mc/PSS2 cells do not appear to be synthesizing PtdSer or PtdEtn at an enhanced rate compared to control McArdle RH7777 cells. These data suggest that PtdSer synthesis from murine PSS1 and PSS2 is differentially regulated. PtdSer synthesis from PSS1 appears to be regulated by a combination of synthesis and degradation, while PtdSer synthesis by PSS2 appears to be regulated by an end-product inhibition mechanism, thus no increase in PtdSer synthesis or mass ensues from expression of murine PSS2..

3.11 Expression of murine PSS2 in McArdle RH7777 cells does not inhibit PtdEtn synthesis from CDP-ethanolamine

It has been previously demonstrated that in McArdle RH7777 cells expressing murine PSS1, there is a dramatic inhibition of the CDP-ethanolamine pathway for PtdEtn biosynthesis (Fig. 3.13)[218]. This finding suggests that the downregulation of the CDP-ethanolamine pathway for PtdEtn biosynthesis represents a compensatory mechanism for the increased production of PtdEtn by decarboxylation of PtdSer in cells expressing murine PSS1 activity. Thus, it appears that this mechanism serves to maintain a constant level of PtdEtn in the cells. Although the *in vitro* serine exchange activity of

McPSS2 cells is 2.5-fold higher than of control cells (Fig. 3.26), the rate of synthesis of PtdSer and PtdEtn was not increased, however (Fig. 3.27). Therefore, when the incorporation of [^{3}H]ethanolamine into PtdEtn was measured in Mc/PSS2 cells, there was no inhibition of PtdEtn synthesis by the CDP-ethanolamine pathway in contrast to what was observed in Mc/PSS1 cells (Fig. 3.30).

3.12 Expression of ET in McArdle RH7777 cells

3.12.1 Expression of human ET cDNA does not increase CDP-ethanolamine or PtdEtn synthesis in McArdle RH7777 cells

In McArdle RH7777 cells expressing murine PSS1 it was demonstrated that there was an increased rate of synthesis of PtdSer and PtdSer-derived PtdEtn, from the decarboxylation of PtdSer (Fig. 3.9), and a dramatic decrease in the synthesis of PtdEtn via the CDP-ethanolamine pathway (Fig. 3.13) as a counter-regulatory mechanism to maintain constant PtdEtn levels within cells [218]. Therefore, it was hypothesized that increased PtdEtn production by the CDP-ethanolamine pathway would inhibit the synthesis of PtdEtn derived from PtdSer.

In order to overproduce PtdEtn by the CDP-ethanolamine pathway, McArdle RH7777 cells were stably transfected with a cDNA isolated from human glioblastoma cells encoding ET [175], the rate-limiting enzyme of the CDP-ethanolamine pathway [169, 170]. Expression of human ET in yeast resulted in an increase in CDP-ethanolamine synthesis in intact cells. In a cell-free assay, ET activity was increased ~3-fold above that in control McArdle RH7777 cells, as measured by the conversion of

[^{3}H]phosphoethanolamine to [^{3}H]CDP-ethanolamine (Fig. 3.31). Control McArdle RH7777 cells and McArdle RH7777 cells expressing human ET were labeled with [^{3}H]ethanolamine for 1 h to determine if the increase in *in vitro* activity corresponded to an increase in the synthesis of CDP-ethanolamine and PtdEtn in intact cells. However, there was no difference in the amount of radiolabel incorporated into the ethanolamine, phosphoethanolamine or CDP-ethanolamine pools, in McArdle RH7777 cells expressing human ET compared to control McArdle RH7777 cells (Fig. 3.32), indicating that increased *in vitro* ET activity did not translate into increased CDP-ethanolamine synthesis, in intact cells. As expected, there was also no increase in the incorporation of labeled ethanolamine into PtdEtn (Fig. 3.33) and no change in the PtdEtn and PtdSer content of these cells (Fig. 3.34). Since there was no increase in PtdEtn synthesis in cells expressing human ET, no change in synthesis of PtdEtn from the decarboxylation of PtdSer was expected. Cells were labeled with [^{3}H]serine for 6 h and the incorporation of label into PtdSer and PtdEtn was measured. There was no difference in the amount of [^{3}H]serine incorporated into PtdEtn in the two cell lines (Fig. 3.35). However, there was a statistically significantly decrease (28%) in the incorporation of [^{3}H]serine into PtdSer in cells stably transfected with human ET, compared to control McArdle RH7777 cells (Fig. 3.35). However, it is unclear why PtdSer synthesis appears to be impaired in McArdle RH7777 cells expressing human ET.

3.12.2 Expression of rat liver ET cDNA increases CDP-ethanolamine, but not PtdEtn, synthesis in McArdle RH7777 cells

Although expression of human ET in yeast resulted in an increase in CDP-ethanolamine synthesis in intact cells, this could not be demonstrated by expression of human ET in McArdle RH7777 cells, even though the *in vitro* activity of ET was increased. The possibility that this cDNA was not a full-length clone was considered, since there is no stop codon present in the 5'- untranslated region. In addition, the cDNA sequence for human ET predicts a protein with a molecular weight of 43.8 kDa, which is significantly smaller than that of purified rat liver ET, which has a molecular mass of 49.5 kDa [171]. Recently, a cDNA from rat liver has been isolated encoding ET (Dr. M. Houweling, unpublished results). This clone was determined to contain the entire coding region of rat liver ET since the predicted amino acid sequence corresponded exactly to N-terminal sequences of purified rat liver ET (Dr. M. Houweling, personal communication). The ET from human also has the same N-terminal sequence as the rat clone, indicating that the human ET cDNA likely contains the complete coding region for ET as well. However, the human ET clone is missing an 18 amino acid sequence between residues 179 and 180, that is present in rat liver ET (Fig. 3.36). Therefore, it is possible that this region is required for the production of CDP-ethanolamine in mammalian cells. Other than the deleted 18 amino acid segment, ET from human and rat liver share a high degree of similarity (Fig. 3.36).

McArdle RH7777 cells were stably transfected with the cDNA for rat liver ET (a gift from Dr. M. Houweling, University of Utrecht, the Netherlands). In a cell free assay,

a ~3-fold increase in ET activity was observed in ET-transfected cells compared to control McArdle RH7777 cells (Fig. 3.37), similar to the expression of human ET (Fig 3.31). The incorporation of [^{3}H]ethanolamine into PtdEtn and the water-soluble intermediates of the CDP-ethanolamine pathway was measured in these cells to determine if the increase in *in vitro* ET activity in these cells corresponded to an increase in the biosynthesis of CDP-ethanolamine and PtdEtn. The incorporation of [^{3}H]ethanolamine into CDP-ethanolamine after 6 h was 15-fold higher in cells expressing rat liver ET compared to control McArdle RH7777 cells (Fig. 3.38, upper panel), which confirms that the cDNA isolated from rat liver does indeed encode ET. Although, a large increase in the amount of radiolabeled CDP-ethanolamine was observed, surprisingly, there was no increase in the amount of radiolabeled ethanolamine incorporated into PtdEtn. Actually, the incorporation of label into PtdEtn was significantly reduced by ~44% (Fig. 3.38, lower panel). To rule out the possibility that PtdEtn synthesis was increased, but the PtdEtn was rapidly degraded, the incorporation of labeled ethanolamine into glycerophosphoethanolamine (a likely degradation product of PtdEtn [161]) was measured, but was found to be unaffected by expression of rat liver ET (Fig. 3.38, upper panel). There was also no difference in the amount of label in ethanolamine and phosphoethanolamine in cells expressing ET and in control cells (Fig. 3.38, upper and lower panel, respectively). This suggests that the increased synthesis of CDP-ethanolamine did not deplete the supply of ethanolamine and phosphoethanolamine.

To determine if increased expression of rat ET decreased the synthesis of PtdEtn from PtdSer, McArdle RH7777 cells expressing rat liver ET and control McArdle

RH7777 cells were labeled with [^{3}H]serine for 4 h and the incorporation of radiolabel into PtdSer and PtdEtn was determined. Unlike the experiments with human ET, there was no significant difference in the amount of [^{3}H]serine incorporated into PtdSer and PtdEtn in McArdle RH7777 cells expressing rat ET compared to that of control McArdle RH7777 cells (Fig. 3.39). These results suggest that increased production of CDP-ethanolamine in McArdle RH7777 cells expressing rat liver ET was not capable of causing a reduction in PtdSer synthesis or PtdEtn synthesis from decarboxylation of PtdSer.

3.13 Tissue distribution of PSS1 and PSS2 in murine tissues

Although several different tissues have been demonstrated to possess PSS activity, it was not known if PSS1 and/or PSS2 contributed to the bulk of total PSS activity in a given tissue. It is possible that the PSS1 and PSS2 genes are differentially expressed, depending on the tissue. Therefore, the tissue distribution of PSS1 and PSS2 in murine tissues was determined by RNA blot analysis. A multiple tissue RNA blot containing 2 μg mRNA isolated from several murine tissues was purchased (Clontech, Palo Alto, CA) and probed with the cDNA encoding murine PSS1, PSS2 and β-actin, as a loading and quality control. RNA blot analysis of murine tissues indicated that PSS1 mRNA was abundant in brain, heart, liver, kidney, testis, with low levels of expression in lung, skeletal muscle and spleen (Fig. 3.40, upper panel). The pattern of PSS2 gene expression was quite different however. PSS2 mRNA was found in testis, was of lower abundance in heart and kidney, and was detectable in liver (Fig. 3.40, middle panel). RNA

blot analysis of β-actin gene expression indicated that the mRNA from these murine tissues was of high quality and there was approximately equal loading of mRNA (Fig. 3.40, lower panel). Since PSS1 and PSS2 are differentially expressed, it is possible that these two proteins have tissue-specific functions, or produce pools of PtdSer for different functions.

Since there appears to be differential expression of the PSS1 and PSS2 genes, it would be important to determine if there was a correlation of gene expression with PSS1 and PSS2 activity. Cellular homogenates of several murine tissues were assayed for choline, ethanolamine and serine base-exchange activities. Testis and kidney possessed the highest serine exchange activity (Fig. 3.41, upper panel) and had the highest mRNA levels for PSS1 and PSS2. Brain and liver had the next highest level of serine exchange activity, but were still dramatically lower than that found in testis and kidney. Examination of the distribution of choline exchange activity, a marker for PSS1, revealed that the highest activity was localized to the brain (Fig. 3.41, middle panel). Although the brain had high choline exchange activity, indicative of PSS1, this did not correspond to a high level of PSS1 gene expression compared to other tissues (Fig. 3.40, upper panel). Testis and kidney by far possessed the highest ethanolamine exchange activity, almost 3-fold higher than any of the other tissues (Fig 3.41, lower panel), which can be correlated with the pattern of PSS2 gene expression (Fig. 3.40, middle panel). This suggests that since brain is enriched in choline exchange activity, with no corresponding enrichment of ethanolamine exchange activity, compared to the other tissues, that PSS1 comprises the majority of the total PSS activity in this tissue. Also, kidney and testis are enriched in

ethanolamine exchange activity without a similar increase in choline exchange activity, compared to the other tissues, suggesting that PSS2 comprises most of the total PSS activity in these two tissues. It is also interesting that although there appears to be very low level of PSS1 gene expression in skeletal muscle, lung and spleen, the in vitro serine exchange activity is not much lower than that of most other tissues examined, indicating that the amount of PSS1 mRNA expressed is not necessarily the limiting factor for PtdSer biosynthesis.

3.14 Subcellular localization of PSS1 and PSS2 in murine liver

Although both PSS1 and PSS2 appear to be expressed in liver, the subcellular distribution of these two enzymes has not been determined in this particular tissue. However, since no anti-PSS1 or anti-PSS2 antibodies are available (see below), the subcellular localization of PSS1 and PSS2 was determined by measuring the ability of unlabeled choline or ethanolamine to inhibit serine exchange activity in ER and MAM subcellular fractions. Since PSS1 is capable of exchanging serine, choline and ethanolamine, unlabeled choline or ethanolamine should be able to compete with radiolabeled serine, in an *in vitro* assay. In contrast, PSS2 can only exchange serine and ethanolamine and therefore, only unlabeled ethanolamine, and not choline, should be able to compete with radiolabeled serine, in an *in vitro* assay. The addition of 5 mM ethanolamine to the PSS assay resulted in a loss of 92% and 85% of the serine exchange activity in ER and MAM, respectively (Fig. 3.42). However, 5 mM choline did not inhibit serine exchange activity in ER, but inhibited 70% of the serine exchange activity in

MAM (Fig. 3.42). These data suggest that ER only contains PSS2, since ethanolamine, but not choline, inhibited serine exchange activity. However, MAM contains PSS1, and possibly PSS2, since both choline and ethanolamine are capable of inhibiting serine exchange activity. Saito *et al.* (1996) provided immunological evidence that in CHO-K1 cells PSS1 was localized to both MAM and microsomes [105], which is not in agreement with the data presented here. However, microsomes are not as homogeneous membrane preparations as the ER fraction isolated from murine liver. Additionally, microsomes usually are contaminated with MAM, which might account for the appearance of PSS1 protein in microsomes.

Since determination of the subcellular localization by measuring base-exchange activity proved partially inconclusive, an alternative approach was undertaken. This involved the production of an antibody against PSS1, which could be used for immunoblot analysis of mouse liver subcellular fractions. Initially, the strategy chosen was to use the cDNA for PSS1 to produce a recombinant PSS1 fusion protein, which could be used as an antigen. An ~800 bp fragment encoding a 31 kDa N-terminal fragment of PSS1 was ligated into the pATH10 expression vector behind the TrpE gene of *E. coli*, which codes for anthranilate synthase. The hybrid protein produced by expression in *E. coli* would then contain 37 kDa of anthranilate synthase and 31 kDa of PSS1. Fig 3.43 (lane 2) shows the protein expression in cells containing the pATH10 vector, producing only the 37 kDa anthranilate synthase fragment, compared to *E. coli*, not expressing any recombinant protein (lane 1) as analyzed by 12% SDS-PAGE. Lanes 3 and 4 show the production of the 68 kDa TrpE/PSS1 fusion protein both under uninduced and induced

conditions (Fig. 3.43). It appears that tryptophan starvation of the cells is sufficient to induce maximal expression, since the addition of indoleacrylic acid to the cells does not further increase the level of expression. This 68 kDa band corresponding to the TrpE/PSS1 fusion protein was excised from the gel and used as an antigen for antibody production in rabbit. However, this approach was unsuccessful because there was no immunogenic response to the antibody identifying PSS1 protein in mouse liver subcellular fractions either at the predicted molecular weight of 55,000 or at the smaller molecular weight observed in CHO-K1 cells, at 42,000.

Additionally, another strategy for the production of an anti-PSS1 antibody was employed. This involved using synthetic peptides based on the predicted amino acid sequence of the PSS1 cDNA from CHO-K1 cells. Peptide 1, containing the sequence, AFTRDDSVPEDN (amino acids 57-68), and peptide 2, containing the sequence, RHSKSKVTNGVGKK (amino acids 458-471) were both linked to keyhole limpet hemocyanin and injected into rabbits. After 2 subsequent booster injections with the peptide antigens, however, no positive immunological response was obtained for either antibody in mouse liver subcellular fractions.

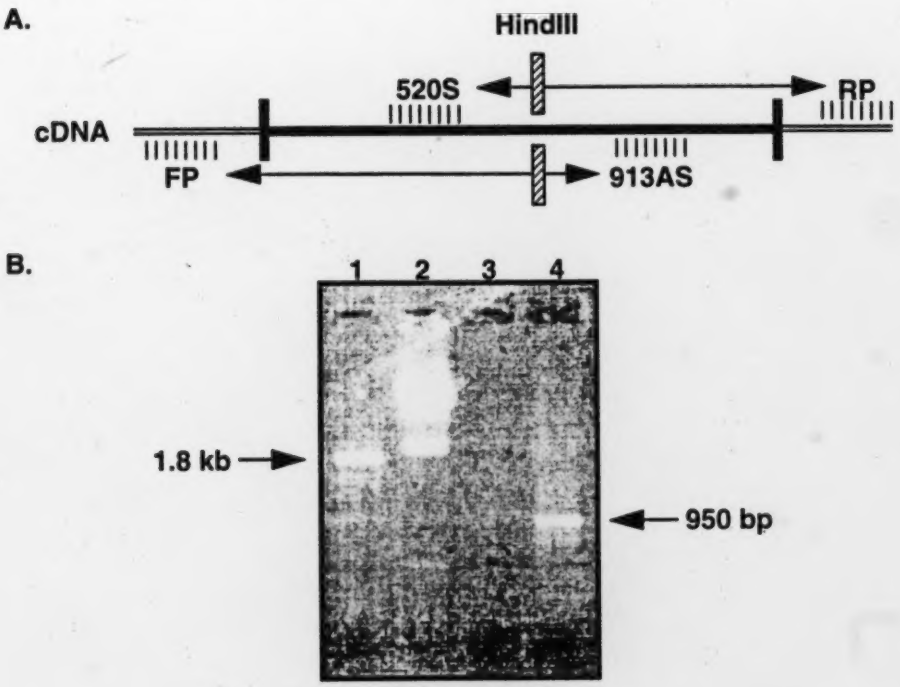


Fig. 3.1. Cloning the cDNA for PSS1 from mouse liver

A) PCR stragedy for cloning PSS1. PCR primers were designed so that a modified λgt11 sequencing primer (FP or RP) would anneal to the λgt11 vector, which contains a mouse liver cDNA library (double line), and PSS1-specific primers (502S or 913AS) would anneal to a homologous region of PSS1 cDNA (solid line). To amplify the 5' end, a λgt11 forward primer (FP) and a PSS1-specific antisense primer (913AS) corresponding to nucleotides 739-764 of CHO-K1 PSS1 was used in a PCR reaction. The 3' end of PSS1 was amplified by PCR using a λgt11 reverse primer (RP) and a PSS1-specific sense primer (520S) from nucleotides 528-555 of CHO-K1 PSS1. The cloning site of the vector is flanked by *EcoRI* restriction sites (solid vertical bars). B) Amplification of the 5' and 3' ends of PSS1 cDNA. Lane 1 shows the PCR product corresponding to the 3' end of the PSS1 cDNA, while lane 4 shows the PCR product corresponding the 5' end. Lane 3 is a PCR reaction carried out in the absence of any DNA template and lane 2 is a λ*HindIII* DNA size ladder.

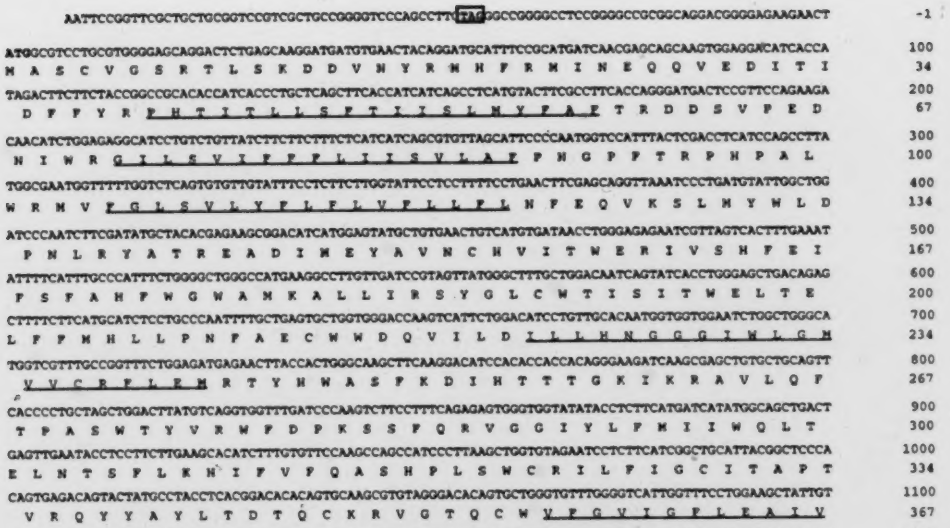


Fig. 3.2. Nucleotide and predicted amino acid sequences of mouse liver PSS1 cDNA.

The putative translational start codon is numbered +1. The first ATG and the stop codon of the largest open reading frame are in boldface. An inframe stop codon (TAG) in the putatative 5' untranslated region is boxed. Six putative transmembrane regions are underlined.

```
CHO     MASCVGSRTLSKDDVNYRMHFRMINEQQVEDITIDFFYRPHTITLLSFTIVSLMYFAFTRDDSVPEDNIWRGILS   75
Mouse   MASCVGSRTLSKDDVNYRMHFRMINEQQVEDITIDFFYRPHTITLLSFTI.SLMYFAFTRDDSVPEDNIWRGILS   75
Human   MASCVGSRTLSKDDVNYKMHFRMINEQQVEDITIDFFYRPHTITLLSFTIVSLMYFAFTRDDSVPEDNIWRGILS   75

CHO     VIFFFLIISVLAFPNGPFTRPHPALWRMVFGLSVLYFLFLVFLLFLNFEQVKSLMYWLDPNLRYATREADIMEYA  150
Mouse   VIFFFLIISVLAFPNGPFTRPHPALWRMVFGLSVLYFLFLVFLLFLNFEQVKSLMYWLDPNLRYATREADIMEYA  150
Human   VIFFFLIISVLAFPNGPFTRPHPALWRMVFGLSVLYFLFLVFLLFLNFEQVKSLMYWLDPNLRYATREAD.MEYA  150

CHO     VNCHVITWERIVSHFDIFAFGHFWGWAMKALLIRSYGLCWTISITWELTELFFMHLLPNFAECWWDQVILDILLC  225
Mouse   VNCHVITWERIVSHF.IF.F.HFWGWAMKALLIRSYGLCWTISITWELTELFFMHLLPNFAECWWDQVILDILL.  225
Human   VNCHVITWERI.SHFDIFAFGHFWGWAMKALLIRSYGLCWTISITWELTELFFMHLLPNFAECWWDQVILDILLC  225

CHO     NGGGIWLGMVVCRFLEMRTYHWASFKDIHTTTGKIKRAVLQFTPASWTYVRWFDPKSSFQRVAGVYLFMIIWQLT  300
Mouse   NGGGIWLGMVVCRFLEMRTYHWASFKDIHTTTGKIKRAVLQFTPASWTYVRWFDPKSSFQRV.G.YLFMIIWQLT  300
Human   NGGGIWLGMVVCRFLEMRTYHWASFKDIHTTTGKIKRAVLQFTPASWTYVRWFDPKSSFQRVAGVYLFMIIWQLT  300

CHO     ELNTFFLKHIFVFQASHPLSWCRILFIGCITAPTVRQYYAYLTDTQCKRVGTQCWVFGVIGFLEAIVCIKFGQDL  375
Mouse   ELNT.FLKHIFVFQASHPLSWCRILFIGCITAPTVRQYYAYLTDTQCKRVGTQCWVFGVIGFLEAIVCIKFGQDL  375
Human   ELNTFFLKHIFVFQASHPLSW.RILFIG.ITAPTVRQYYAYLTDTQCKRVGTQCWVFGVIGFLEAIVCIKFGQDL  375

CHO     FSKTQILYVV.WLLCVAFTTFLCLYGMVWYAEHYGHREKTYSECEDGT--PEISWHHGKGSKGSEDSPPKHSSNN  448
Mouse   FSKTQILYV.LWLLCVAFTTFLCLYGMVWYAEHYGHREKTYSECEDGTYSPEISWHHGKGSKGSEDSPPKHSSN.  450
Human   FSKTQILYVV.WLLCVAFTTFLCLYGM.WYAEHYGHREKTYSECEDGTYSPEISWHH.KG.KGSEDSPPKH..NN  450

CHO     ESHSSRRRNRHSKSKVTNGVGKK                                                    471
Mouse   ESHSSRRRNRHSKSKVTNGVGKK                                                    473
Human   ESHSSRRRNRHSKSKVTNGVGKK                                                    473
```

Fig. 3.3. Comparison of the predicted amino acid sequences of PSS1 from CHO-K1 cells, human myeloblastic cells and mouse liver.

Sequences of PSS1 from CHO-K1 and human myeblastic cells (Genbank accession # D14694) were aligned with the deduced amino acid sequence of mouse liver PSS1. Non-identical amino acids are indicated by dots. Gaps in the sequences are indicated by dashes.

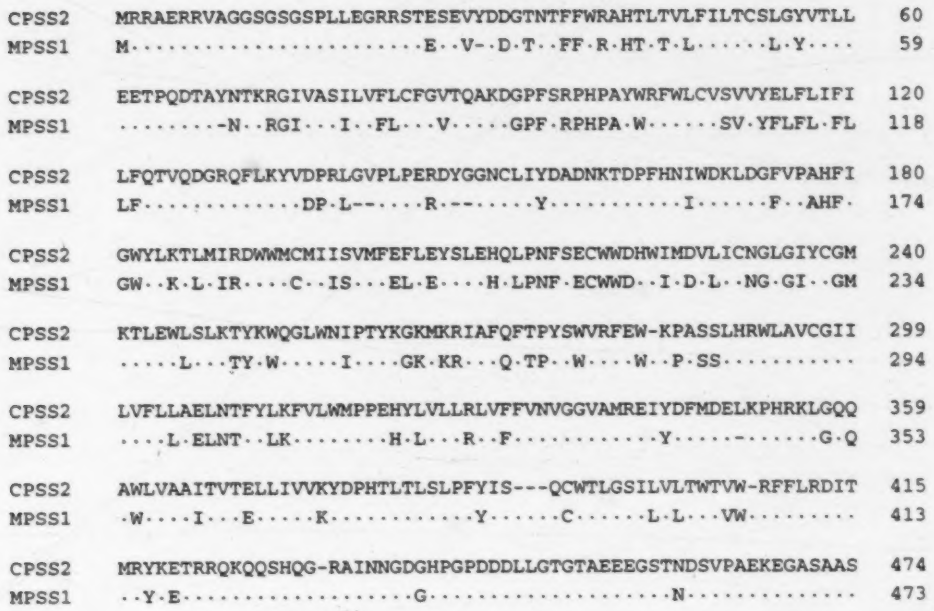


Fig. 3.4. Comparison of the predicted amino acid sequences of PSS1 from murine liver and PSS2 from CHO-K1 cells.

The deduced amino acid sequence sequences encdoed by PSS2 from CHO-K1 cells (CPSS2) and PSS1 from mouse liver (MPSS1) were aligned. Non-identical amino acids are indicated by dots. Gaps in the amino acid sequences are indicated by dashes.

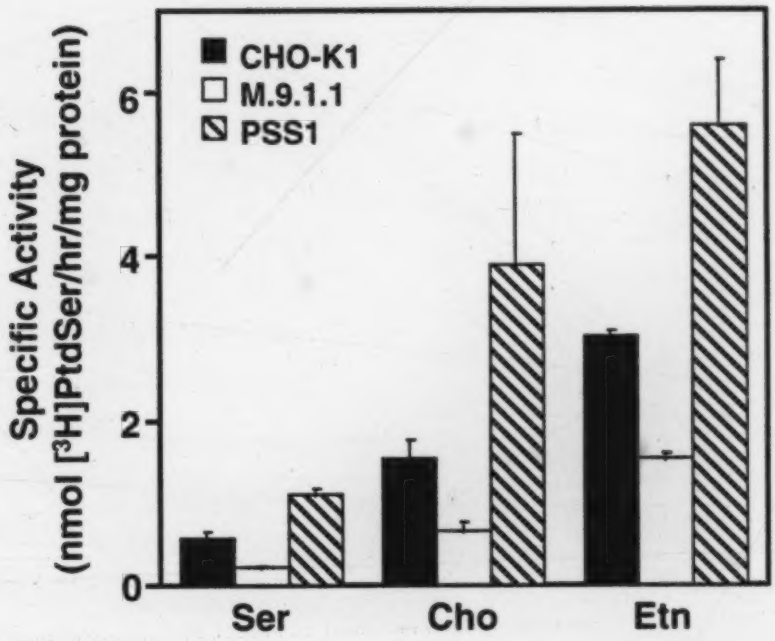


Fig. 3.5. Base-exchange enzyme activities of wild type CHO-K1 cells, M.9.1.1 cells and control M.9.1.1 cells expressing mouse liver PSS1.

Base exchange activities were measured using [3-^{3}H]serine (Ser), [1-^{3}H]ethanolamine (Etn) and [*methyl*-^{3}H]choline (Cho) in cellular lysates from CHO-K1 cells (solid bars), control M.9.1.1 cells transfected with empty vector (open bars), and M.9.1.1 cells expressing PSS1 cDNA (hatched bars). Data are averages ± S.D. of triplicate analyses from one experiment that was representative of 3 similar experiments.

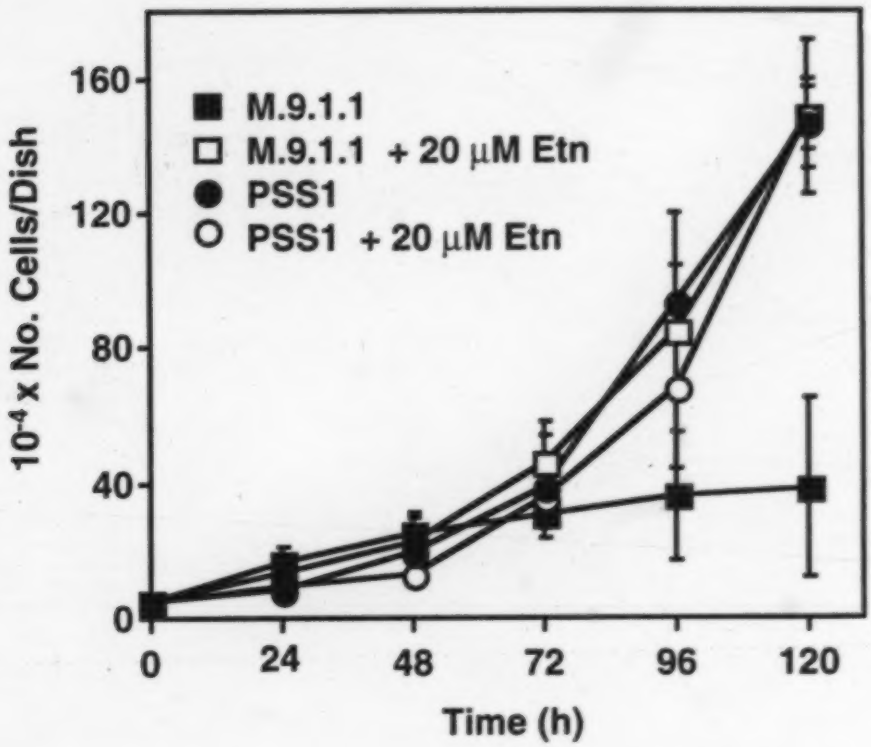


Fig. 3.6. Growth of M.9.1.1 cells and M.9.1.1 cells expressing mouse liver PSS1. Control M.9.1.1 cells (squares) and M.9.1.1 cells expressing mouse PSS1 cells (circles) were plated at a density of 5 x 10^4 cells/60 mm dish and cultured in Ham's F-12 medium supplemented with 10% delipidated fetal bovine serum with (open symbols) or without (solid symbols) 20 μM ethanolamine. Cells were harvested at 24 h intervals by trypsinization and counted. Data are averages ± S.D. of triplicate analyses from 3 experiments. Some error bars are hidden by the symbols.

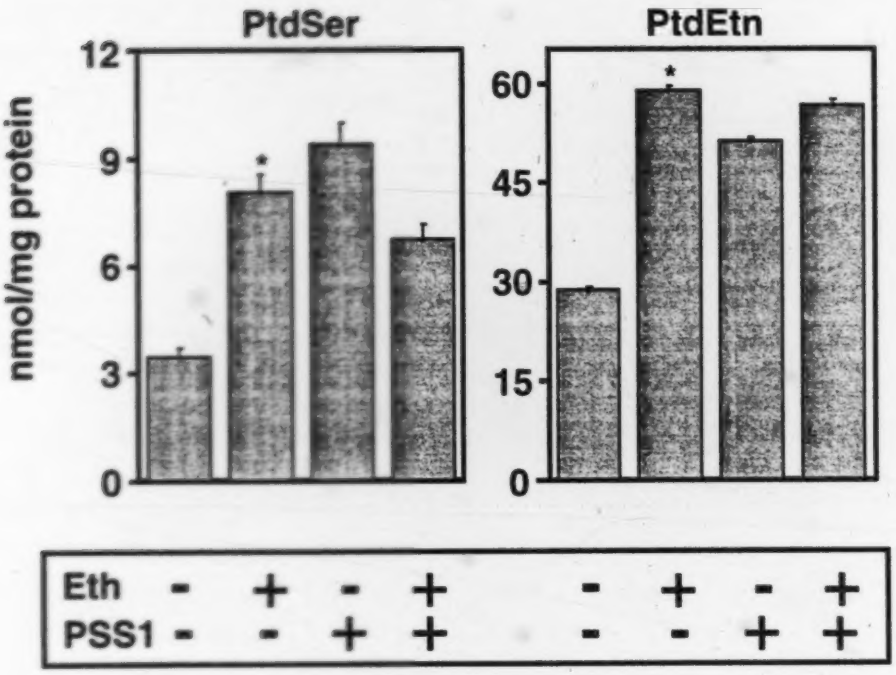


Fig. 3.7. PtdSer and PtdEtn content of control M.9.1.1 cells and M.9.1.1 cells expressing mouse liver PSS1.

Cells were grown in medium supplemented with 20 μM ethanolamine for 48 h. Medium with (+) or without (-) 20 μM ethanolamine (Eth) was then added for an additional 48 h after which cells were harvested and the content of PtdSer and PtdEtn was determined. PSS1 (+) denotes M.9.1.1 cells transfected with PSS1 cDNA; PSS1 (-) denotes control M.9.1.1 cells. Data are averages ± S.D. of triplicate analyses from 3 independent experiments. *Statistical significance ($p < 0.05$) of differences between cells cultured in the presence and absence of 20 μM ethanolamine was evaluated by the Student's t test.

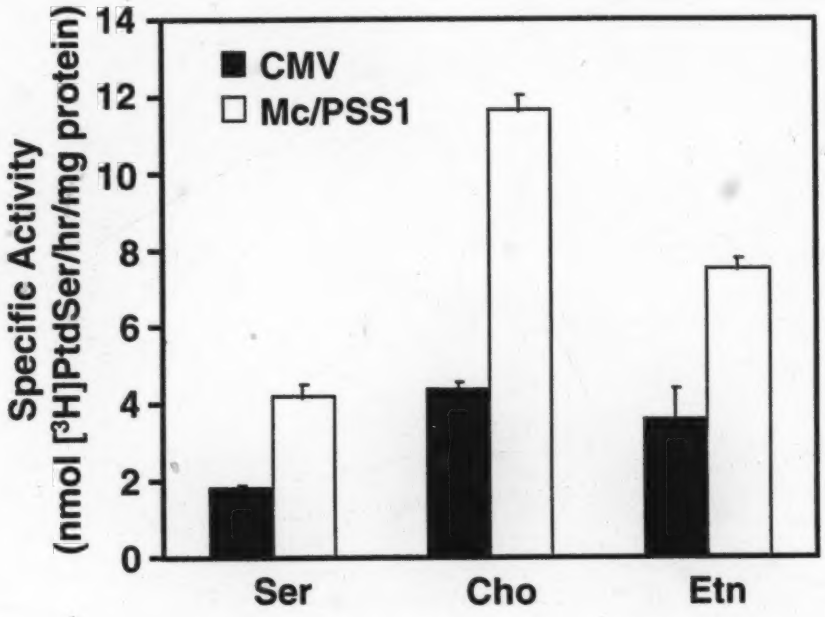


Fig. 3.8. Base-exchange activities of control McArdle RH7777 cells and McArdle RH7777 cells expressing mouse liver PSS1 (Mc/PSS1).

Base exchange activities were measured in cellular lysates using [3-^{3}H]serine (Ser), [*methyl*-^{3}H]choline (Cho) and [1-^{3}H]ethanolamine (Etn). Control McArdle RH7777 cells (solid symbols); Mc/PSS1 cells (open symbols). Data are averages ± S.D. of triplicate analyses from 3 independent experiments.

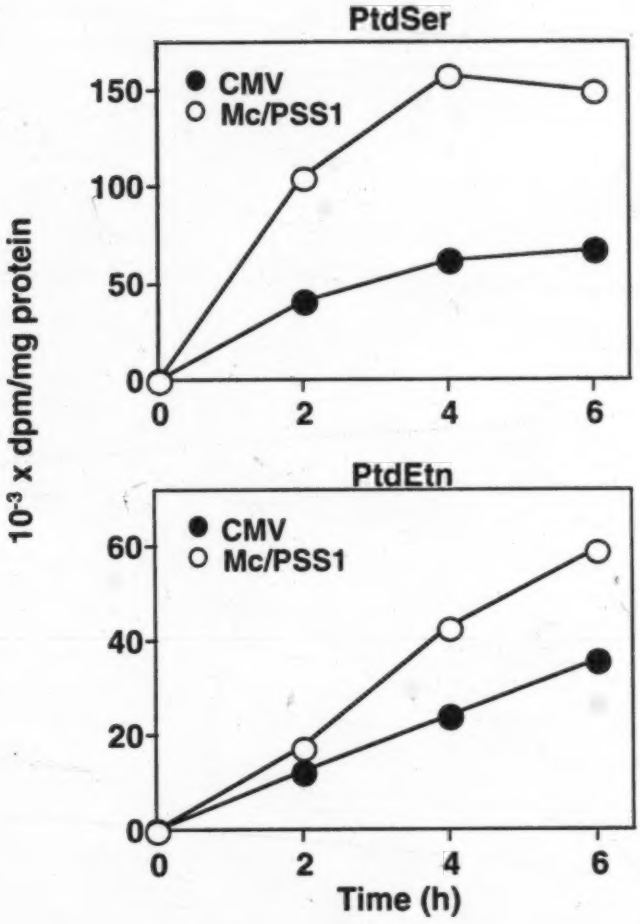


Fig. 3.9. Mc/PSS1 cells have an increased rate of incorporation of [^{3}H]serine into PtdSer and PtdEtn compared to control cells.

Cells were incubated in medium containing [3-^{3}H]serine (20 μCi/dish). At the indicated times, cells were harvested and PtdSer and PtdEtn were extracted and separated by thin-layer chromatography. Control McArdle RH7777 cells (CMV, solid symbols); Mc/PSS1 cells (open symbols). Data are averages ± S.D. of triplicate analyses from 3 independent experiments. Error bars are obscured by the symbols.

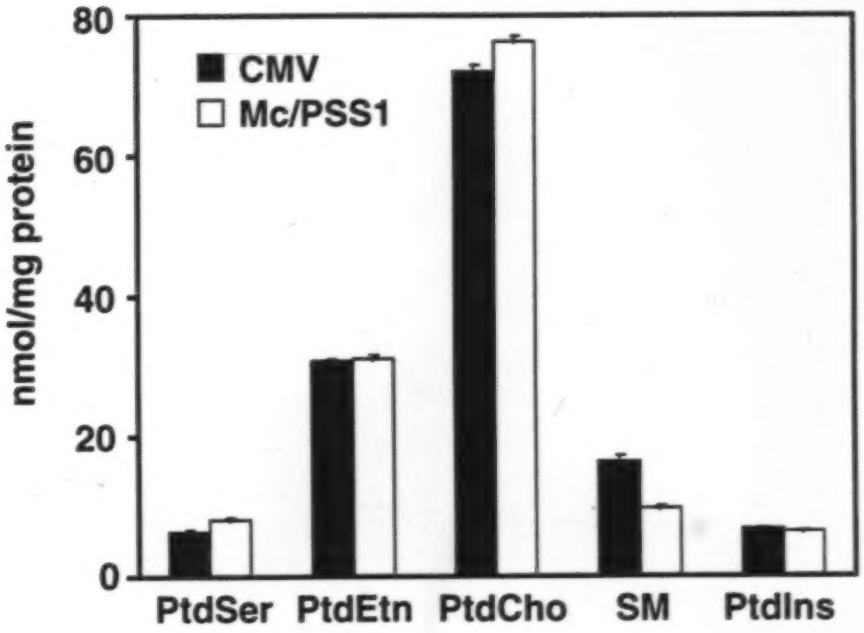


Fig. 3.10. The phospholipid composition of Mc/PSS1 cells is not altered.

Cells were grown to 80% confluence then harvested and the phospholipids were extracted and separated by thin-layer chromatography. The phosphorus content of PtdSer, PtdEtn, phosphatidylcholine (PtdCho), sphingomyelin (SM) and phosphatidylinositol (PtdIns) was determined. Control McArdle RH7777 cells (CMV, solid symbols); Mc/PSS1 cells (open symbols). Data are averages ± S.D. of triplicate analyses from 3 independent experiments.

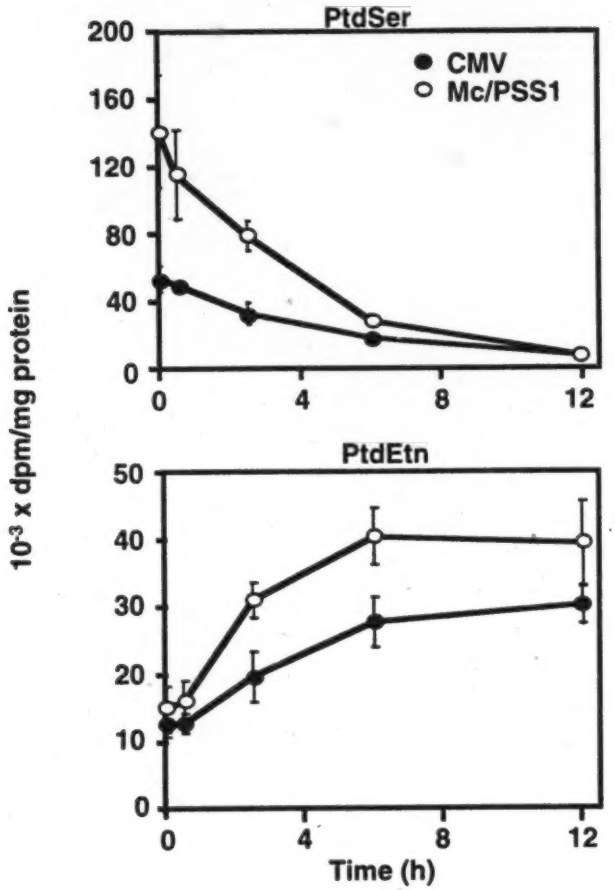
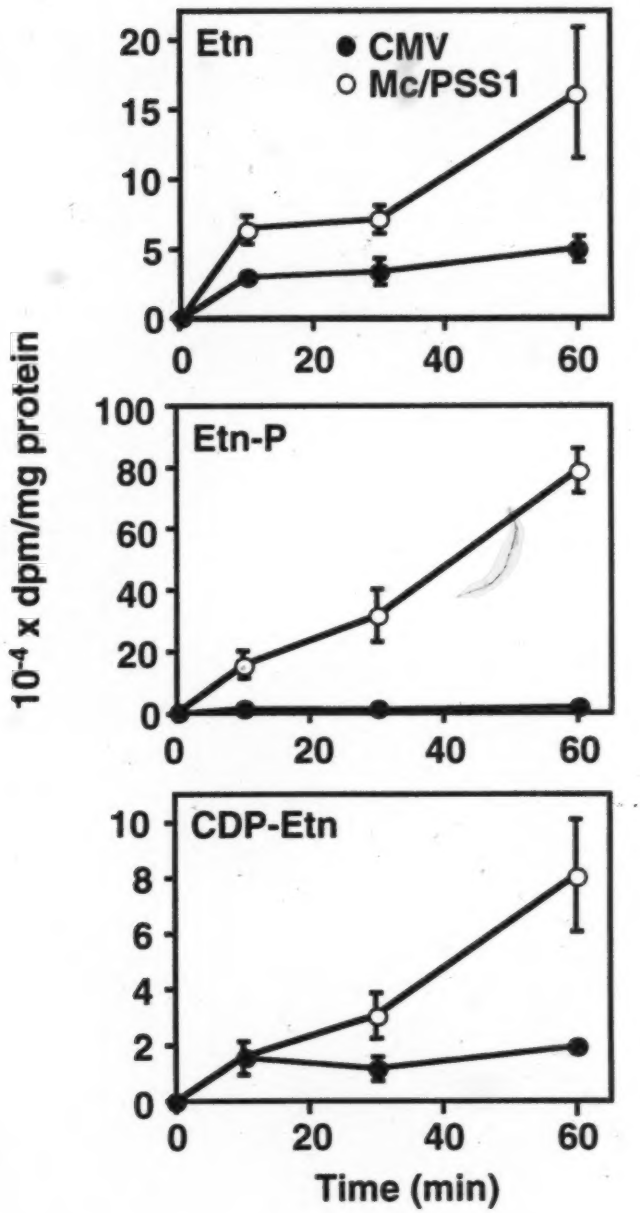


Fig. 3.11. The rate of turnover/degradation of [^{3}H]serine-derived PtdSer and PtdEtn in control McArdle RH7777 cells and Mc/PSS1 cells is increased.

Cells were incubated for 1 h in medium containing [^{3}H]serine (20 μCi/dish). The medium was then removed and fresh medium containing 1 mM unlabeled serine was added. At the indicated times cells were harvested and PtdSer (upper panel) and PtdEtn (lower panel) were extracted and separated by thin-layer chromatography. Control McArdle RH7777 cells (CMV, solid symbols); Mc/PSS1 cells (open symbols). Data are averages ± S.D. from triplicate analyses from one experiment which was repeated twice with similar results. Some error bars are too small to be visible.

Fig. 3.12. [^{3}H]ethanolamine-derived metabolites of the CDP-ethanolamine pathway accumulate in Mc/PSS1 cells.

Cells were incubated in medium containing [^{3}H]ethanolamine (1.5 μCi/dish) for the indicated times and the amount of radioactivity incorporated into the water-soluble intermediates of the CDP-ethanolamine pathway (Etn = ethanolamine, Etn-P = phosphoethanolamine, CDP-Etn = CDP-ethanolamine) was determined. Control McArdle RH7777 cells (CMV, solid symbols); Mc/PSS1 cells (open symbols). Data are averages ± S.D. of triplicate analyses from one experiment which was repeated twice with similar results. Some error bars are obscured by symbols.


Etn
CMV
Mc/PSS1
Etn-P
CDP-Etn
10^{-4} x dpm/mg protein
Time (min)

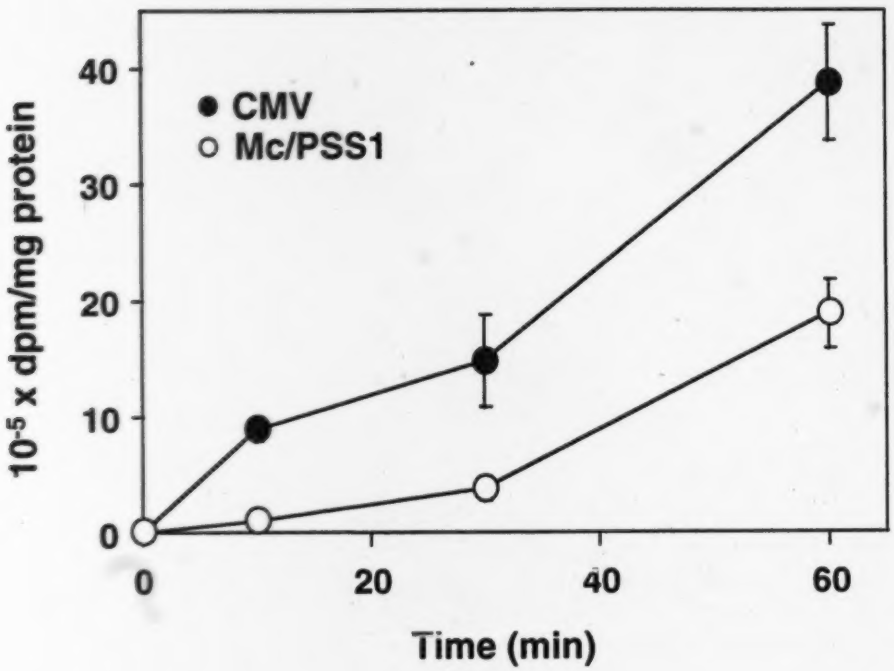


Fig. 3.13. Rate of incorporation of [^{3}H]ethanolamine into PtdEtn is lower in Mc/PSS1 cells.

Cells were incubated in medium containing [^{3}H]ethanolamine (1.5 μCi/dish) for the indicated times and the amount of radioactivity in PtdEtn was determined. Control McArdle RH7777 cells (CMV, solid symbols); Mc/PSS1 cells (open symbols). Data are averages ± S.D. of triplicate analyses from 3 experiments. Some error bars are obscured by symbols.

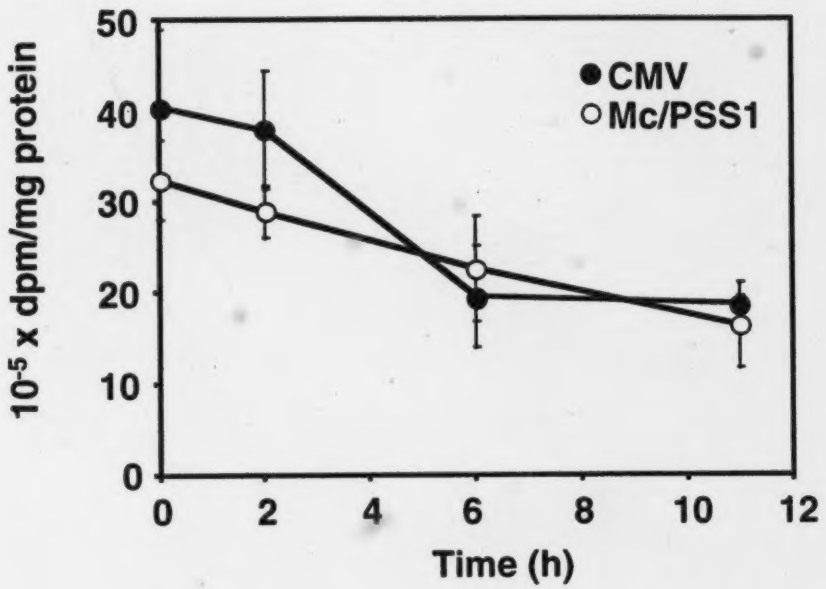


Fig. 3.14. Rate of degradation of [^{3}H]ethanolamine-derived PtdEtn is not increased in Mc/PSS1 cells.

Control McArdle RH7777 cells (CMV, closed symbols) and Mc/PSS1 cells (open symbols) were labeled with [^{3}H]ethanolamine (1.5 μCi/dish) for 24 h. The radiolabeled medium was removed and replaced with medium containing (2 mM) unlabeled ethanolamine. Cells were harvested at the indicated times and incorporation of radiolabel into PtdEtn was determined. Data are averages ± S.D. of triplicate analyses from one experiment which was repeated twice with similar results.

	Specific Activity	
	Control	Mc/PSS1
	nmol/min/mg protein	
Enzyme		
EK	**2.00 ±0.44**	**1.60 ±0.44**
ET[a] (-i)	**0.12 ±0.02**	**0.12 ±0.05**
ET[a] (+i)	**0.09 ±0.03**	**0.14 ±0.01**
EPT (+d)	**0.65 ±0.24**	**0.63 ±0.22**
EPT (-d)	**0.12 ±0.02**	**0.11 ±0.01**

[a]Data are averages ± S.D. of triplicate analysis from a single experiment

Table 1. The enzyme activities of the CDP-ethanolamine pathway are not reduced in Mc/PSS1 cells.

Total cellular membranes and cytosol were prepared from control McArdle RH7777 cells and Mc/PSS1 cells by centrifugation of cell lysates at 400,000 x *g* for 30 minutes. Cytosol was assayed for EK and ET activities. Membranes were assayed for EPT activity. The data for the EK and EPT assays are averages from triplicate analyses of 2 or 3 experiments. ET activity was assayed in triplicate in the presence (+i) or absence (-i) of 5 μM microcystin, 1mM sodium vanadate, and 10 mM NaF. EPT activity was measured in the presence (+d) and absence (-d) of exogenous diacylglycerol.

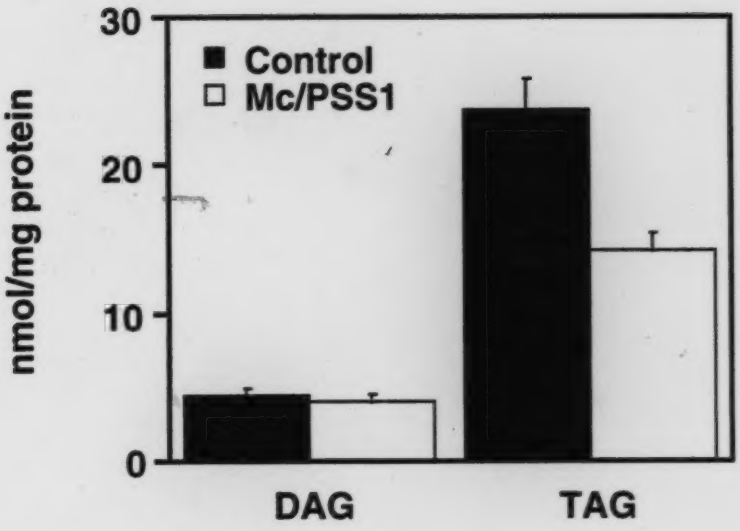
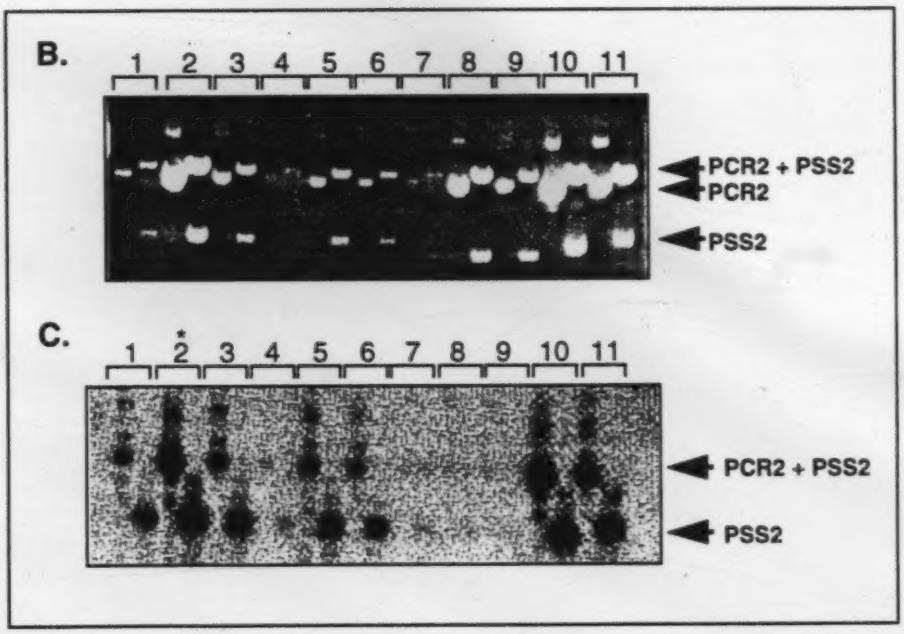


Fig. 3.15. Triacylglycerol, but not diacylglycerol, levels are reduced in Mc/PSS1 cells.

Control McArdle RH7777 cells and Mc/PSS1 cells were harvested at approximately 80% confluency. Lipids were extracted and separated by thin-layer chromatography and the amount of TAG and DAG was measured. Data are averages ± S.D. of triplicate analyses from 2 independent experiments.

Fig. 3.16. Cloning the cDNA for PSS2 from mouse liver by PCR.

A) The coding region for PSS2 was amplified from a λgt11 cDNA library by PCR using primers based on the PSS2 cDNA isolated from CHO-K1 cells. The sense primer contained the initiation codon, ATG, and the antisense primer contained the stop codon, TGA. PCR products were separated by agarose gel electrophoresis. Lane 1 is a PCR reaction carried out in the absence of any DNA template; lane 2 is a λ/ *HindIII* DNA size ladder, lane 3 shows the products of a PCR reaction containing DNA template. B) A 1.4 kB band was excised from the gel, the DNA was extracted and ligated into the PCR2 cloning vector. cDNA was isolated from 11 different clones and digested with *EcoRI* and separated by agarose gel electrophoresis. C) The DNA was transferred to a nylon membrane and probed with a PSS2-specific oligonucleotide. The left lane in each bracket is undigested DNA, the right lane is DNA digested with *EcoRI*. Arrows indicate DNA fragements corresponding to inserts released from the PCR2 vector by restriction digestion with *EcoRI*. An asterisk indicates the clone containing PSS2 cDNA used for subsequent experiments.


A.
1 2 3
1.4 kb
B.
1 2 3 4 5 6 7 8 9 10 11
PCR2 + PSS2
PCR2
PSS2
C.
1 2 3 4 5 6 7 8 9 10 11
PCR2 + PSS2
PSS2

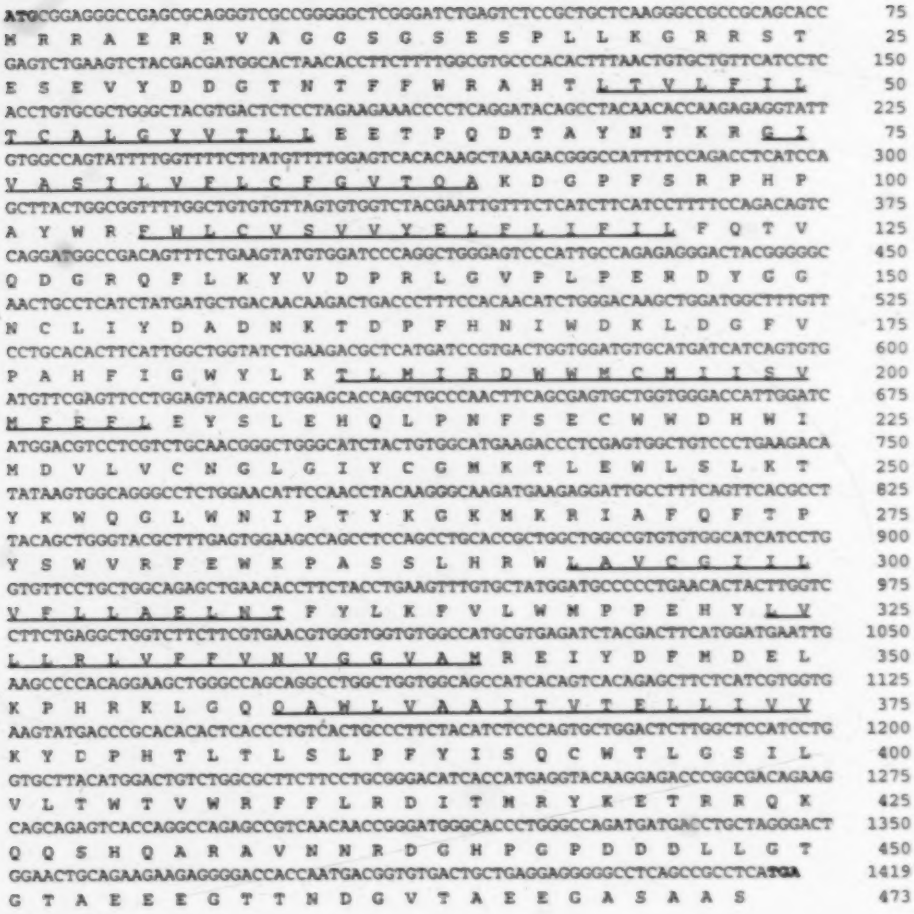


Fig. 3.17. Nucleotide and predicted amino acid sequences of murine liver PSS2 cDNA.

The translational start codon is numbered +1. The start codon, ATG, and the stop codon (TGA) of the largest open reading frame are in boldface. Seven putative transmembrane regions are underlined.

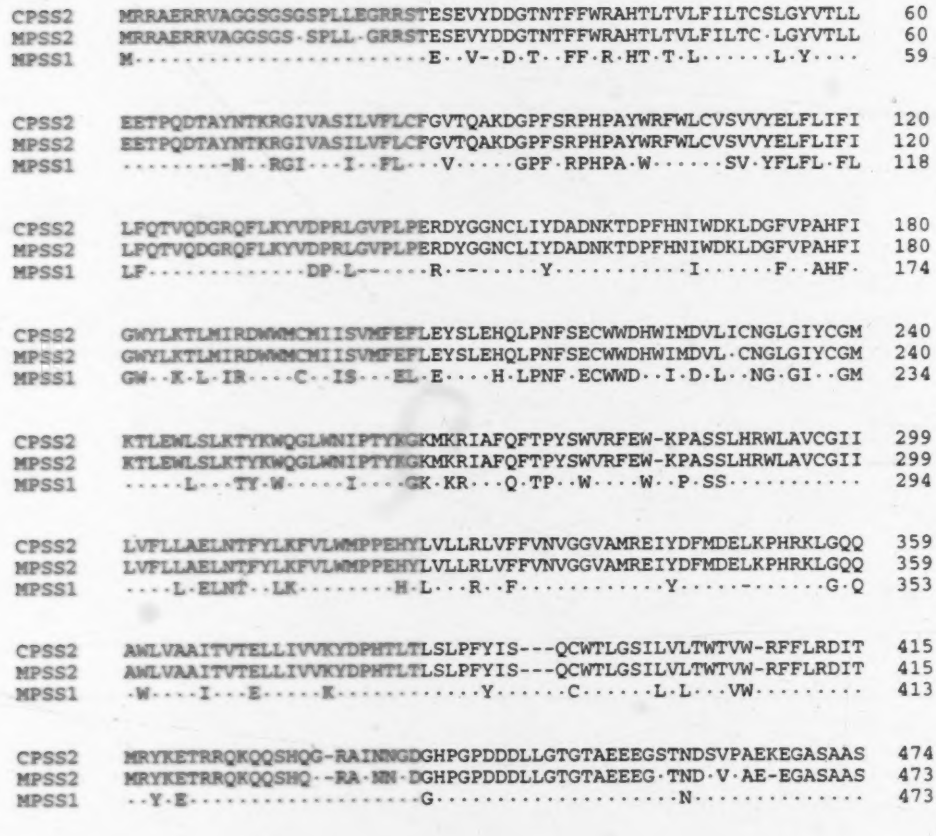


Fig. 3.18. Comparison of the predicted amino acid sequences of PSS1 and -2 from mouse liver and PSS2 from CHO-K1 cells.

Sequences of PSS2 from CHO-K1 cells (CPSS2) and PSS1 from mouse liver (MPSS1) were aligned with the deduced amino acid sequence of mouse liver PSS2 (MPSS2). Non-identical amino acids are indicated by dots. Gaps in the amino acid sequences are indicated by dashes.

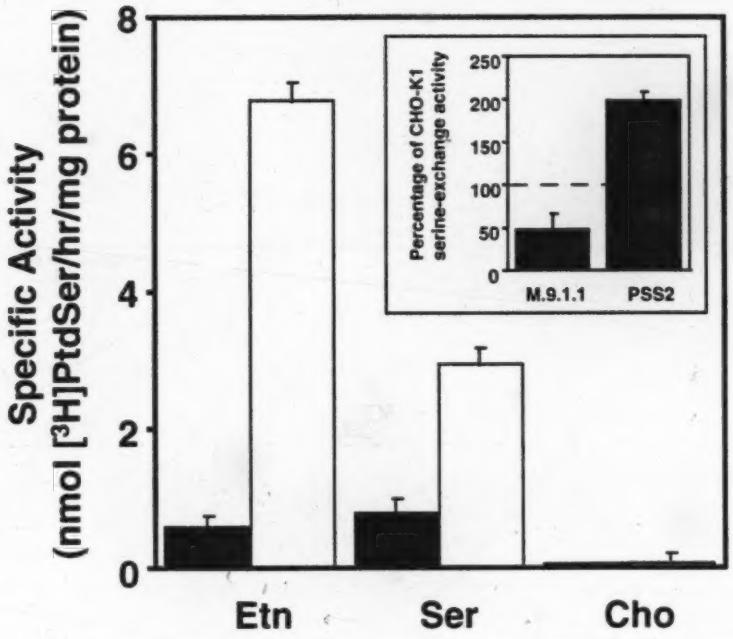


Fig. 3.19. Base-exchange enzyme activities of control M.9.1.1 cells and M.9.1.1 cells expressing mouse liver PSS2.

Base exchange activities were measured using [3-^{3}H]serine (Ser), [1-^{3}H]ethanolamine (Etn) and [*methyl*-^{3}H]choline (Cho) in cellular lysates from control M.9.1.1 cells (solid bars), and M.9.1.1 cells transfected with murine PSS2 cDNA (open bars). Inset shows serine-exchange activity of M.9.1.1 cells and M.9.1.1. cells expressing PSS2 relative to wild-type CHO-K1 cells. Data are averages ± S.D. of triplicate analyses from 3 independent experiments.

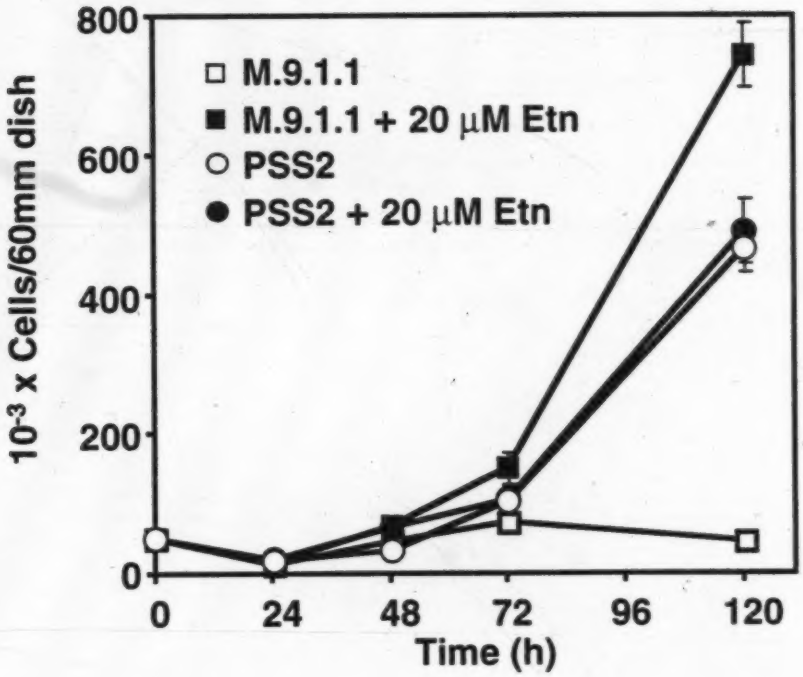


Fig. 3.20. Expression of murine PSS2 cDNA in M.9.1.1 cells complements the growth defect of this cell line.

Control M.9.1.1 cells (squares) and M.9.1.1 cells transfected with murine PSS2 cDNA (circles) were plated at a density of 5 x 10^4 cells/60 mm dish and cultured in Ham's F-12 medium supplemented with 10% delipidated fetal bovine serum with (solid symbols) or without (open symbols) 20 μM ethanolamine. Cells were harvested at 24 h intervals by trypsinization and counted. Data are averages ± S.D. of triplicate analyses from one experiment which was repeated once with similar results. Some error bars are hidden by the symbols.

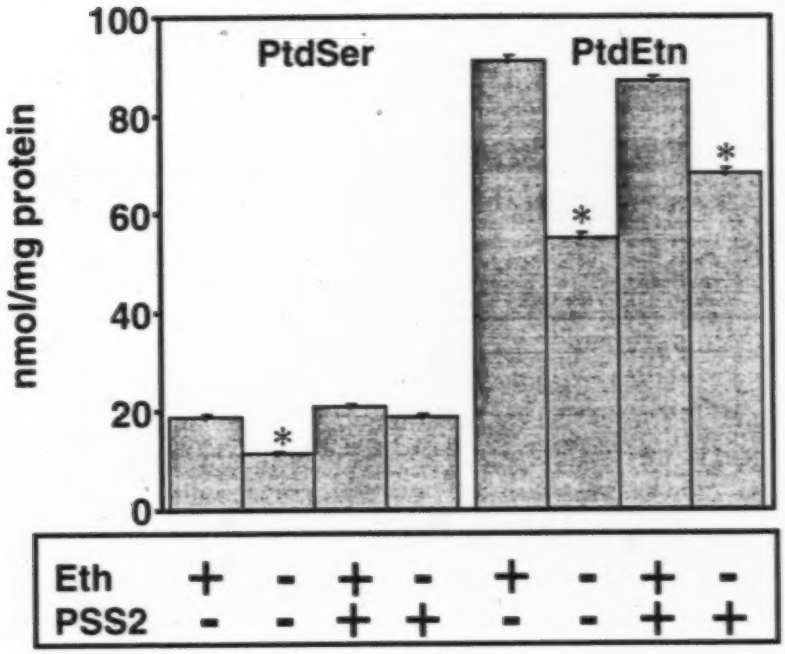


Fig. 3.21. Expression of PSS2 cDNA in M.9.1.1 cells restores the PtdSer, but not PtdEtn, content of M.9.1.1 cells.

Cells were grown in medium supplemented with 20 μM ethanolamine for 24 h. Medium with (+) or without (-) 20 μM ethanolamine (Eth) was then added for an additional 72 h after which cells were harvested and the content of PtdSer and PtdEtn was determined. PSS2 (+) denotes M.9.1.1 cells transfected with murine PSS2 cDNA; PSS2 (-) denotes control M.9.1.1 cells. Data are averages ± S.D. of triplicate analyses from 3 independent experiments. *Statistical significance ($p < 0.05$) of differences between cells cultured in the presence and absence of 20 μM ethanolamine was evaluated by the Student's t test.

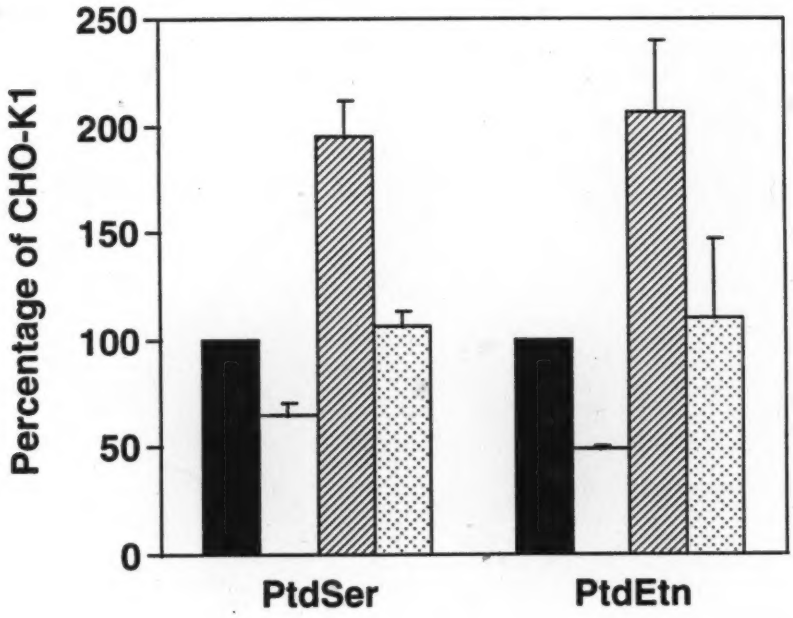


Fig. 3.22. Incorporation of [^{3}H]serine into PtdSer and PtdEtn of control M.9.1.1 cells and M.9.1.1 cells expressing murine PSS1 or PSS2.

Cells were incubated in medium containing [3-^{3}H]serine (15 μCi/dish) for 6 h, after which the radioactivity incorporated into PtdSer and PtdEtn was determined. Data are expressed as the amount of [3-^{3}H]serine incorporated into PtdSer and PtdEtn relative to CHO-K1 cells. Solid bars, CHO-K1; open bars, M.9.1.1; hatched bars, M.9.1.1 cells expressing PSS1; stippled bars, M.9.1.1 cells expressing PSS2. Data are averages ± S.D. of triplicate analyses from 3 experiments.

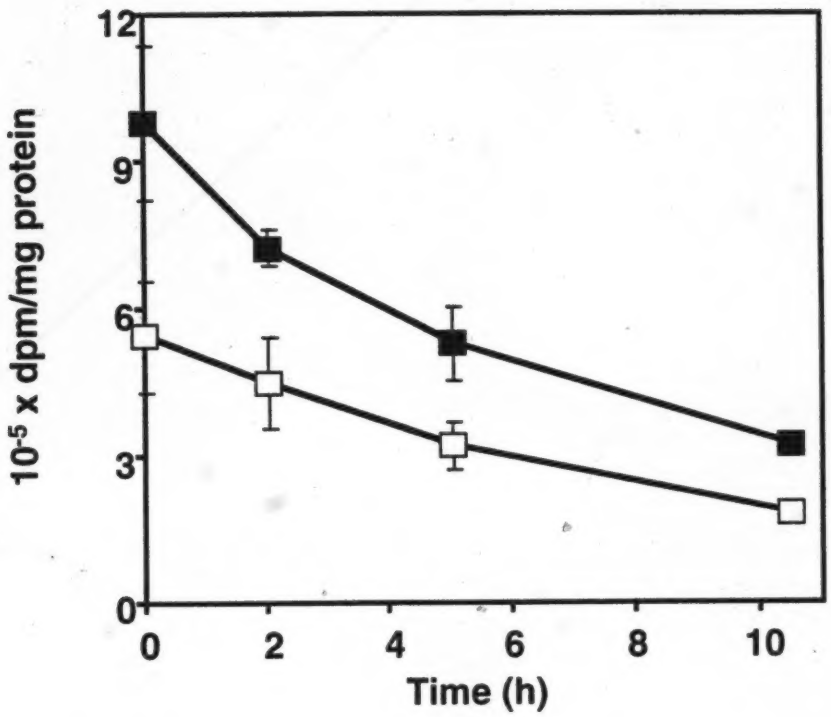


Fig. 3.23. The turnover of [^{3}H]ethanolamine-derived PtdEtn in M.9.1.1 cells expressing murine PSS2 is not altered.

Cells were incubated in medium containing [^{3}H]ethanolamine (1.5 μCi/dish) for 6 h after which the medium was removed and replaced with fresh medium containing 2 mM unlabled ethanolamine. Cells were then harvested and phospholipids were isolated. Solid symbols, control cells; open symbols, M.9.1.1 cells expressing PSS2. Data are averages ± S.D. of triplicate analyses from one experiment which was repeated once with similar results.

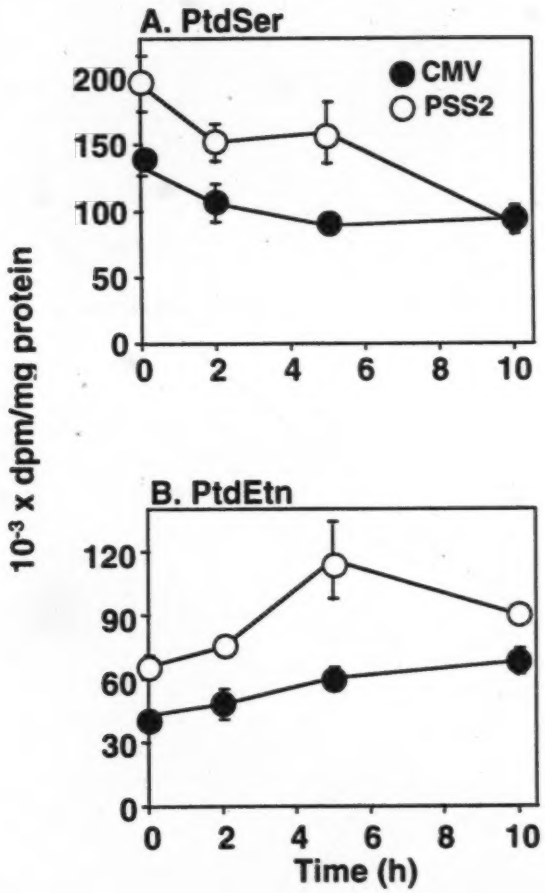


Fig. 3.24. Metabolism of [^{3}H]serine-derived PtdSer and PtdEtn in control M.9.1.1 cells and M.9.1.1 cells transfected with murine PSS2.

Cells were incubated for 6 h in medium containing [3-^{3}H]serine (15 μCi/dish) after which the medium was removed and replaced with fresh medium containing 2 mM unlabled serine. At the indicated times cells were harvested and PtdSer (panel A) and PtdEtn (Panel B) were isolated. Closed symbols, control M.9.1.1 cells; open symbols, M.9.1.1 cells expressing PSS2. Data are averages ± S.D. of triplicate analyses from one experiment which was repeated twice with similar results.

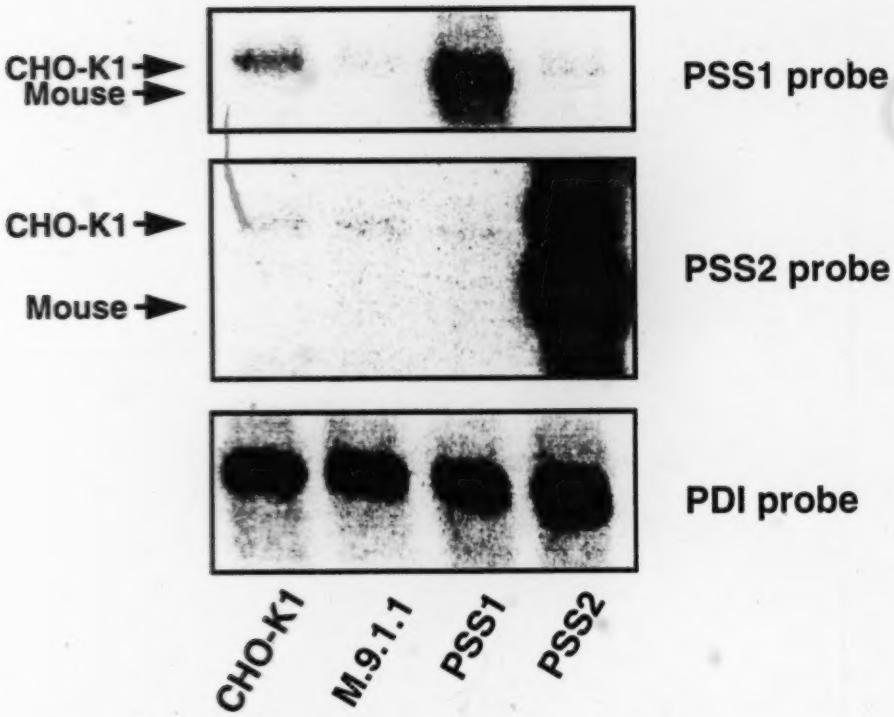


Fig. 3.25. Northern blot analysis of PSS1 and PSS2 mRNAs.

5 µg of poly $(A)^+$ RNA was separated on a 1.2% agarose/formaldehyde gel and transferred to a Hybond N membrane. PSS1, PSS2 and protein disulfide isomerase (PDI) cDNA probes were hybridized at 42 °C in the presence of 50% formamide. Washes were performed at the following stringencies: PSS1, 1x SSC at 60 °C; PSS2, 0.1 x SSC at 60 °C; PDI, 0.1x SSC at 55 °C. Upper panel, PSS1 cDNA probe; middle panel, PSS2 cDNA probe; lower panel, PDI cDNA probe. The RNA samples are indicated at the bottom of the figure and were from CHO-K1 cells, control M.9.1.1 cells and M.9.1.1 cells transfected with PSS1 (PSS1) or PSS2 (PSS2).

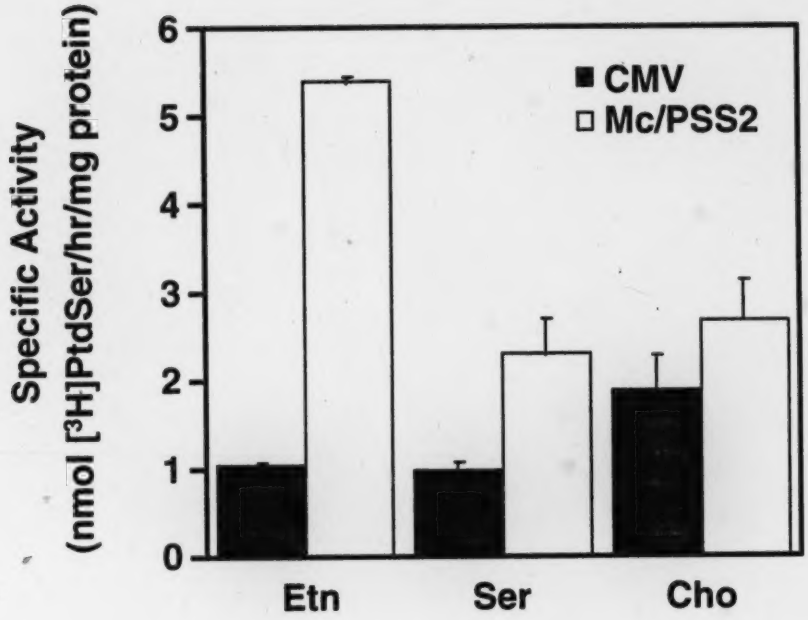


Fig. 3.26. Base-exchange activities of control McArdle RH7777 cells and McArdle RH7777 cells expressing murine PSS2 (Mc/PSS2).

Base exchange activities were measured in cellular lysates using [3-^{3}H]serine (Ser), [*methyl*-^{3}H]choline (Cho) and [1-^{3}H]ethanolamine (Etn). Control McArdle RH7777 cells (CMV, solid bars); Mc/PSS2 cells (open bars). Data are averages ± S.D. of triplicate analyses from 3 independent experiments.

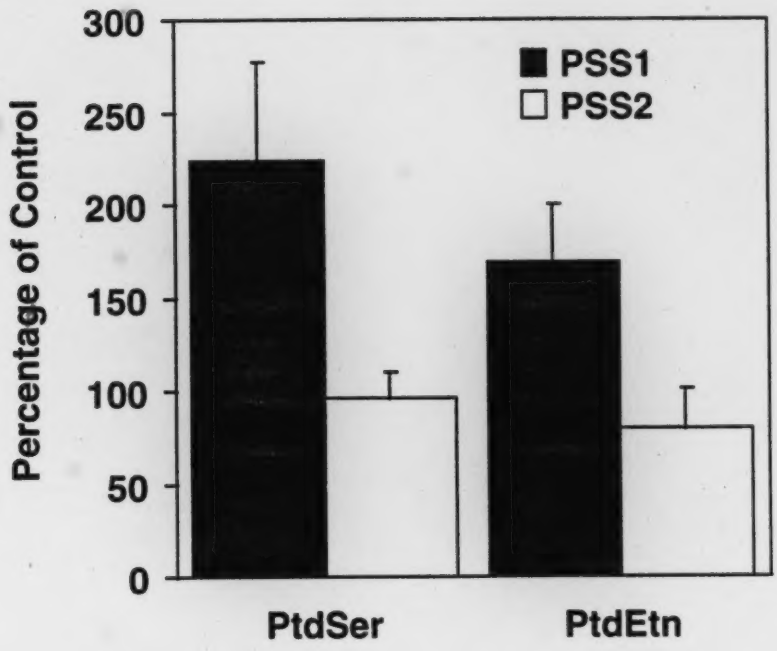


Fig. 3.27. McArdle RH7777 cells expressing murine PSS1, but not PSS2, have more [^{3}H]serine incorporated into PtdSer and PtdEtn compared to control McArdle cells.

Cells were incubated in medium containing [3-^{3}H]serine (15 μCi/dish) and harvested after 6 h. The radioactivity in PtdSer and PtdEtn was then determined. Data are expressed as the amount of radiolabel incorporated into PtdSer and PtdEtn in McArdle RH7777 cells expressing murine PSS1 and PSS2 relative to control McArdle RH7777 cells. McArdle RH7777 cells expressing murine PSS1 (solid bars); McArdle RH7777 cells expressing murine PSS2 (open bars). Data are averages ± S.D. of triplicate analyses from 3 experiments.

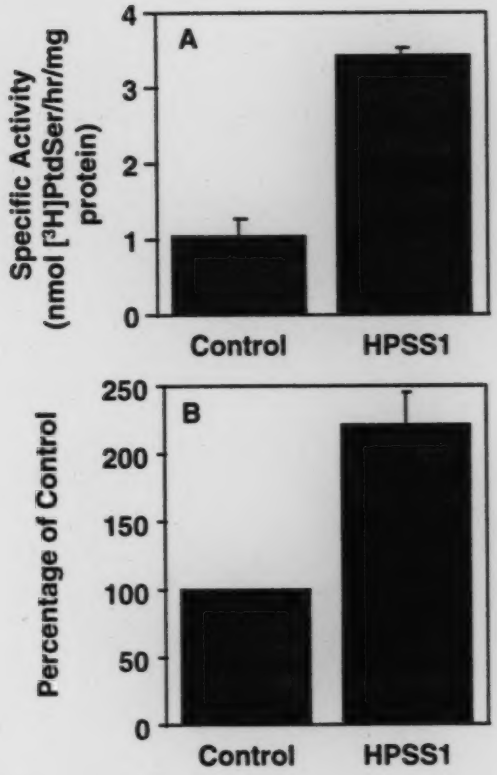


Fig. 3.28. Expression of human PSS1 in McArdle RH7777 cells increases serine exchange activity and increases the incorporation of [^{3}H]serine into PtdSer compared to control cells.

A) Serine base exchange activities were measured in cellular lysates of control McArdle RH7777 cells and McArdle RH7777 cells expressing human PSS1 (HPSS1). Data are averages ± S.D. of triplicate analyses from 2 experiments. B) Incorporation of [^{3}H]serine into PtdSer and PtdEtn of control cells and McArdle RH7777 cells expressing human PSS1. Cells were incubated in medium containing [3-^{3}H]serine (20 μCi/dish) for 6 h and then the amount of radioactivity in PtdSer and PtdEtn was determined. Data are averages ± S.D. of duplicate analyses from 3 experiments and are expressed as percentage of control.

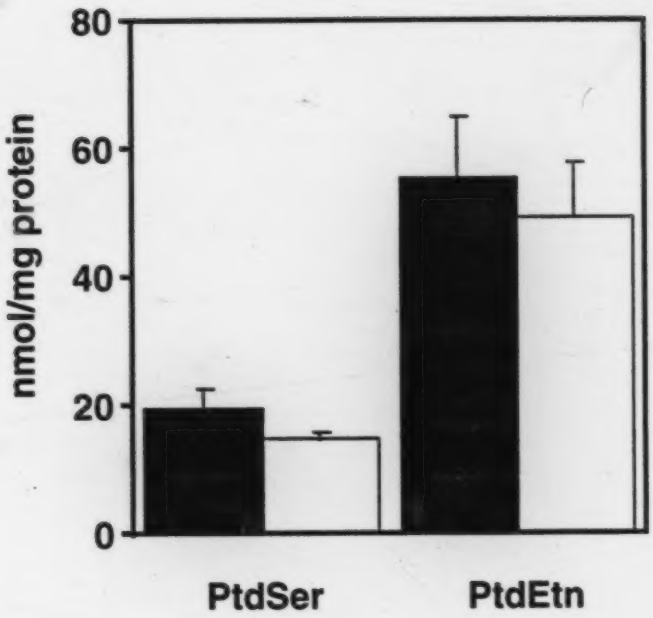


Fig. 3.29. PtdSer and PtdEtn content McArdle RH7777 cells expressing murine PSS2 is not altered, compared to control McArdle RH7777 cells.

Cells were grown to 80% confluence then harvested and the phospholipids were extracted and separated by thin-layer chromatography. The phosphorus content of PtdSer and PtdEtn was determined. Control McArdle RH7777 cells (solid bars); Mc/PSS2 cells (open bars). Data are averages ± S.D. of triplicate analyses from 2 independent experiments.

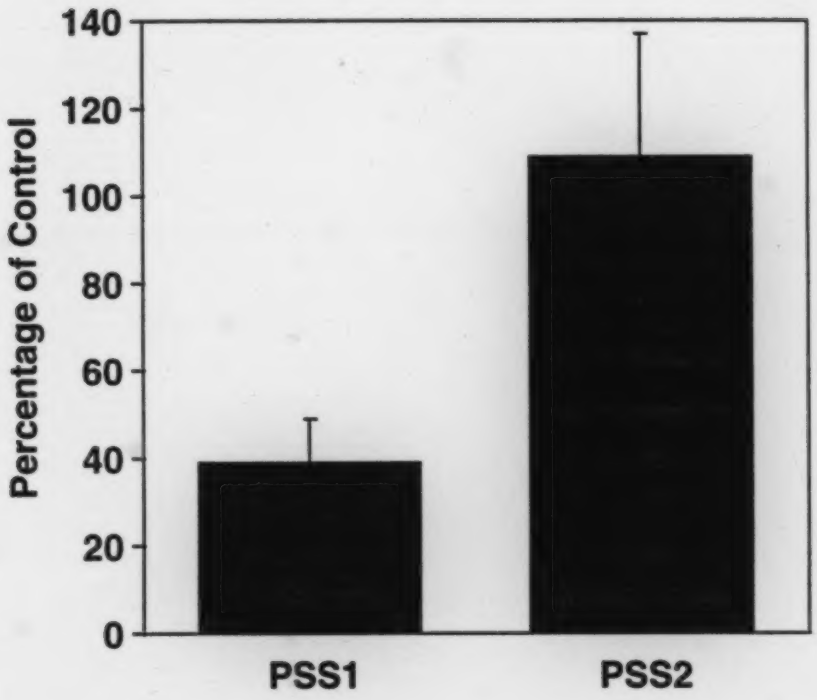


Fig. 3.30. McArdle RH7777 cells expressing murine PSS2, do not have reduced incorporation of [^{3}H]ethanolamine into PtdEtn compared to control McArdle RH7777 cells.

Cells were incubated in medium containing [^{3}H]ethanolamine (1.5 μCi/dish) for 1 h. Cells were then harvested and phospholipids were isolated. Data are expressed as the amount of radiolabel incorporated into PtdEtn in McArdle RH7777 cells expressing murine PSS1 or PSS2 relative to that in control McArdle RH7777 cells. Data are averages ± S.D. of triplicate analyses from two experiments.

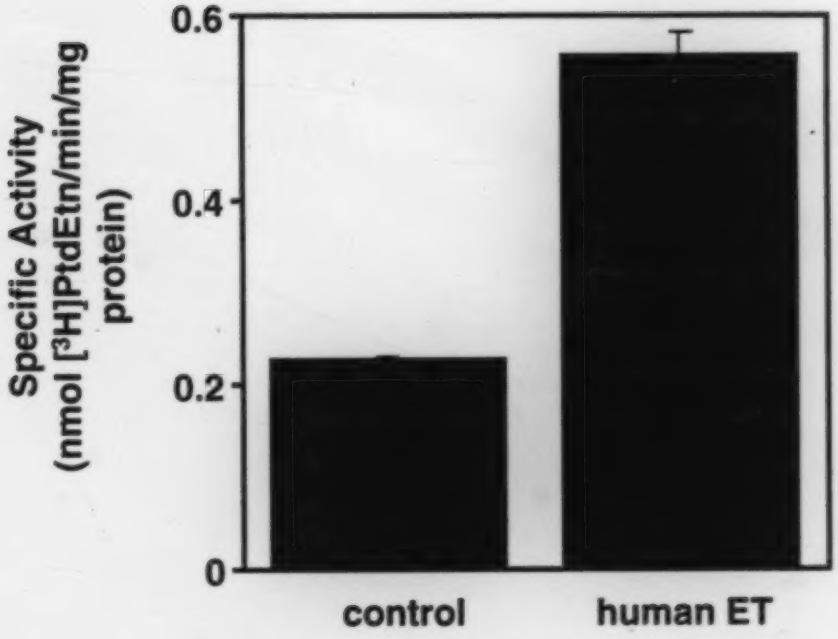
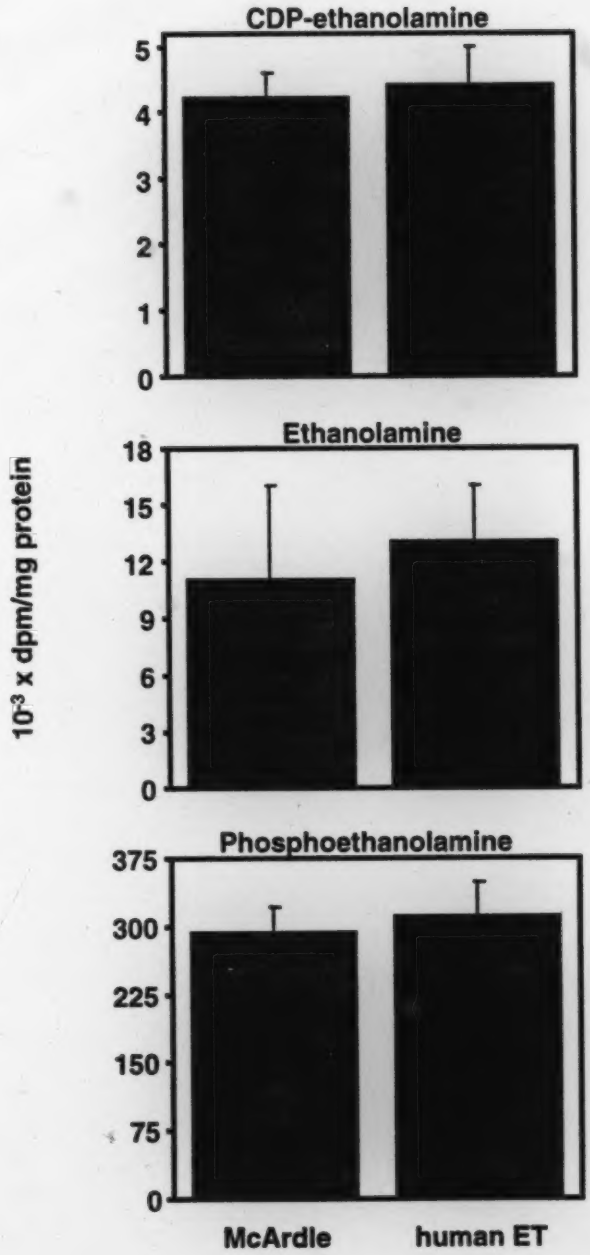


Fig. 3.31. Expression of human ET in McArdle RH7777 cells.

ET activity was measured in cellular lysates of control McArdle RH7777 cells and McArdle RH7777 cells expressing human ET using [^{3}H]phosphoethanolamine. Data are averages ± S.D. of triplicate analyses from 3 independent experiments.

Fig. 3.32. McArdle RH7777 cells expressing human ET do not contain more [^{3}H]CDP-ethanolamine than control McArdle RH7777 cells.

Control McArdle RH7777 cells and McArdle RH7777 cells expressing human ET were incubated in medium containing [^{3}H]ethanolamine (1.5 μCi/dish). After 6h, cells were harvested and the amount of radioactivity in the water soluble intermediates of the CDP-ethanolamine pathway was determined. Data are averages ± S.D. of triplicate analyses from one experiment.


CDP-ethanolamine
5
4
3
2
1
0
Ethanolamine
18
15
12
9
6
3
0
10-3 x dpm/mg protein
Phosphoethanolamine
375
300
225
150
75
0
McArdle
human ET

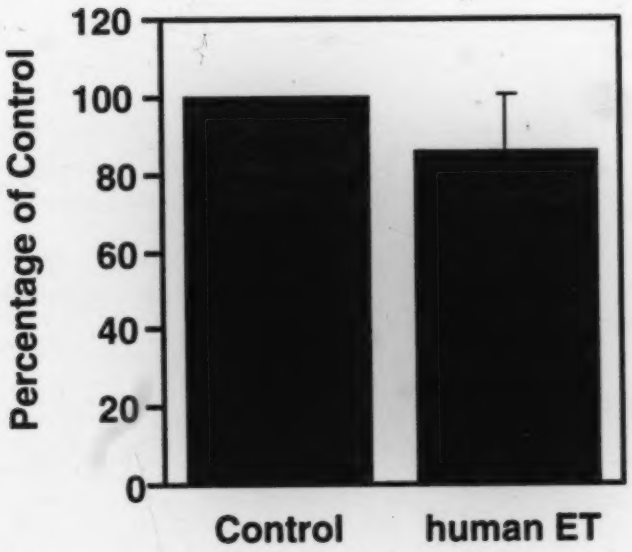


Fig. 3.33. McArdle RH7777 cells expressing human ET do not incorporate more [^{3}H]ethanolamine into PtdEtn compared to control cells.

Control McArdle RH7777 cells and McArdle RH7777 cells expressing human ET were incubated in medium containing [^{3}H]ethanolamine (1.5 μCi/dish). After 6h, cells were harvested and the amount of radioactivity in the lipid extract was determined. Data are averages ± S.D. of triplicate analyses from 3 experiments and are expressed as a percentage of control.

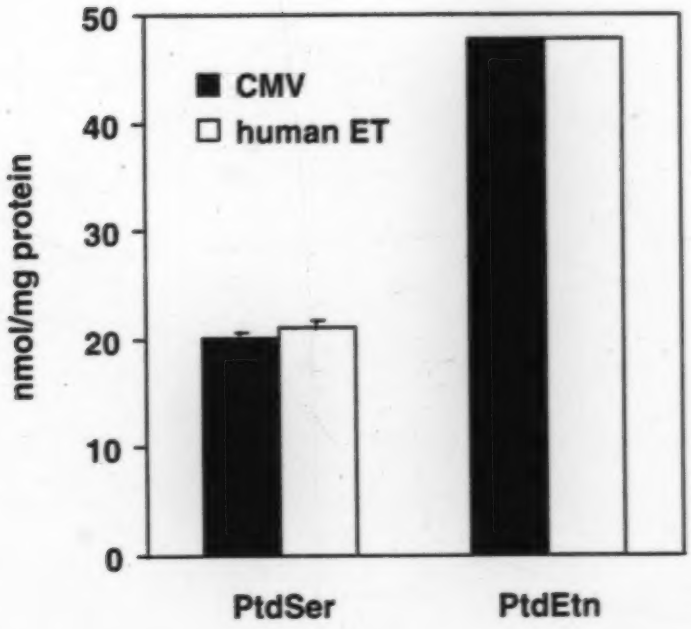


Fig. 3.34. The PtdSer and PtdEtn content of McArdle RH7777 cells expressing human ET is not altered compared to control cells.

Cells were grown to 80% confluence then harvested and the phospholipids were extracted and separated by thin-layer chromatography. The phosphorus content of PtdSer and PtdEtn was determined. Control McArdle RH7777 cells (CMV, solid symbols); McArdle RH7777 cells transfected with human ET cDNA (open symbols). Data are averages ± S.D. of triplicate analyses from 2 independent experiments.

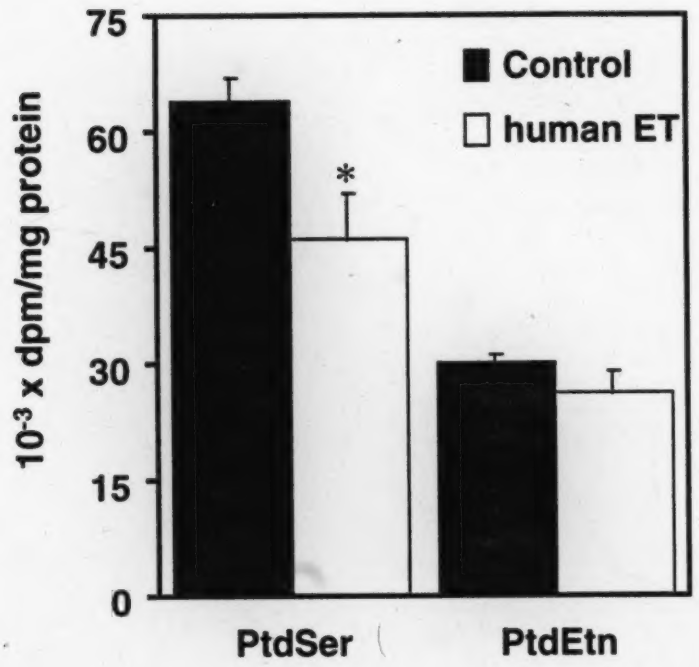


Fig. 3.35. Incorporation of [^{3}H]serine into PtdSer and PtdEtn of McArdle RH7777 cells and McArdle RH7777 cells expressing human ET.

Control RH7777 McArdle cells and McArdle RH7777 cells expressing human ET were incubated in medium containing [3-^{3}H]serine (20 μCi/dish) for 6 h. Cells were harvested and PtdSer and PtdEtn were extracted and separated by thin-layer chromatographyand the radioactivity in each lipid determined. Data are averages ± S.D. of triplicate analyses from one experiment which was repeated twice with similar results. *Statistical significance ($p < 0.05$) of differences between control McArdle RH7777 cells and McArdle RH7777 cells expressing human ET was evaluated by the Student's t test.

```
rat     MIRNGHGAGGAAGLKGPGGQRTVRVWCDGCYDMVHYGHSNQLRQARAMGD     50
human   MIRNG.GA.G.A...GPGG.R.VRVWCDGCYDMVHYGHSNQLRQARAMGD     50

rat     YLIVGVHTDEEIAKHKGPPVFTQEERYKMVQAIKWVDEVVPAAPYVTTLE    100
human   YLIVGV.TDEEIAKHKGPPVFTQEERYKMVQAIKWVDEVVPAAPYVTTLE    100

rat     TLDKHNCDFCVHGNDITLTVDGRDTYEEVKQAGRYRECKRTQGVSTTDLV    150
human   TLDK.NCDFCVHGNDITLTVDGRDTYEEVKQAGRYRECKRTQGVSTTDLV    150

rat     GRMLLVTKAHHSSQEMSSEYREYADSFGKPPHPTPAGDTLSSEVSSQCPG    200
human   GRMLLVTKAHHSSQEMSSEYREYADSFGK----------------CPG    182

rat     GQSPWTGVSQFLQTSQKIIQFASGKEPQPGETVIYVAGAFDLFHIGHVDF    250
human   G..PWTGVSQFLQTSQKIIQFASGKEPQPGETVIYVAGAFDLFHIGHVDF    232

rat     LQEVHKLAKRPYVIAGLHFDQEVNRYKGKNYPIMNLHERTLSVLACRYVS    300
human   L..VH.LA.RPY.IAGLHFDQEVN.YKGKNYPIMNLHERTLSVLACRYVS    282

rat     EVVIGAPYSVTAELLNHFKVDLVCHGKTEIVPDRDGSDPYEEPKRRGIFC    350
human   EVVIGAPY.VTAELL.HFKVDLVCHGKTEI.PDRDGSDPY.EPKRRGIFR    332

rat     QIDSGSDLTTDLIVQRIIKNRLEYEARNQKKEAKELAFLEALRQQEAQPR    400
human   QIDSGS.LTTDLIVQRII.NRLEYEARNQKKEAKELAFLEA.RQQ.AQP.    382

rat     GETD---                                            404
human   GE.DGDF                                            389
```

Fig. 3.36 Comparison of the predicted amino acid sequences of human and rat liver ET.

The predicted amino acid sequences of ET from a human glioblastoma cell lines and from rat liver were aligned. Non-identical amino acids are indicated by dots. Gaps in the amino acid sequences are indicated by dashes.

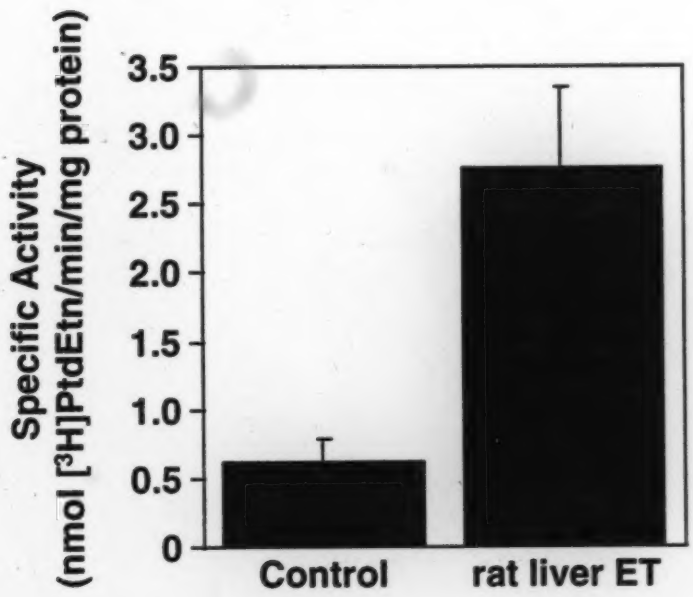


Fig. 3.37. Expression of rat liver ET in McArdle RH7777 cells.

ET activity was measured in cellular lysates of control RH7777 McArdle cells (control) and McArdle RH7777 cells expressing rat liver ET using [^{3}H]phosphoethanolamine. Data are averages ± S.D. of duplicate analyses from 2 experiments.

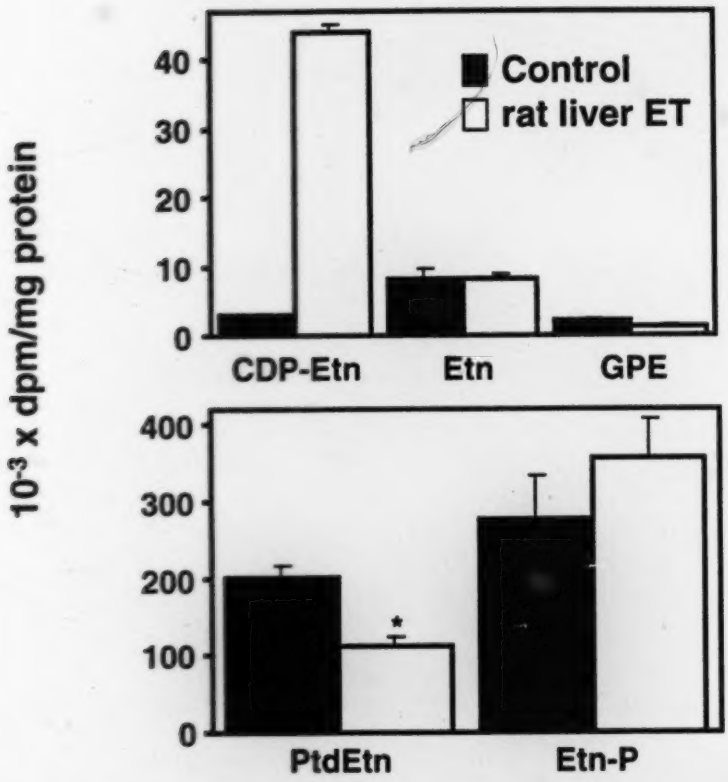


Fig. 3.38. McArdle RH7777 cells expressing rat liver ET have increased content of [^{3}H]CDP-ethanolamine, but not PtdEtn, compared to control McArdle RH7777 cells.

Cells were incubated in medium containing [^{3}H]ethanolamine (3 μCi/dish). After 4 h, cells were harvested and the amount of radioactivity in PtdEtn and the water soluble intermediates of the CDP-ethanolamine pathway, as well as glycerophosphoethanolamine, was determined. Ethanolamine, Etn; phosphoethanolamine, Etn-P, CDP-ethanolamine, CDP-Etn; glycerophosphoethanolamine, GPE. Data are averages ± S.D. of triplicate analyses from one experiment which was repeated twice with similar results. *Statistical significance ($p < 0.005$) of differences between control McArdle RH7777 cells and McArdle RH7777 cells expressing rat liver ET was evaluated by the Student's t test.

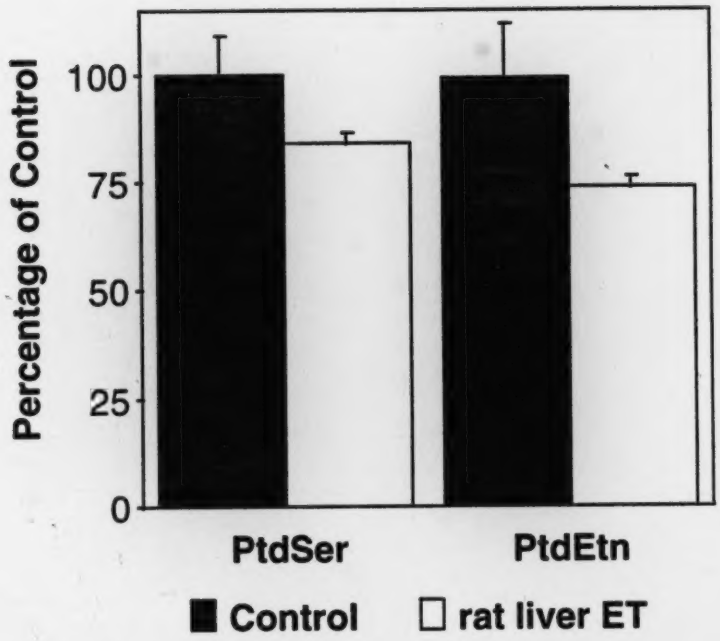


Fig. 3.39. Incorporation of [^{3}H]serine into PtdSer and PtdEtn of McArdle RH7777 cells and McArdle RH7777 cells expressing rat liver ET.

Cells were incubated in medium containing [3-^{3}H]serine (20 μCi/dish). At the indicated times, cells were harvested and PtdSer and PtdEtn were extracted and separated by thin-layer chromatography. Control McArdle RH7777 cells, solid bars, McArdle RH7777 cells expressing rat liver ET, open bars. Data are averages ± S.D. of triplicate analyses from 2 experiments and are expressed as percentage of control.

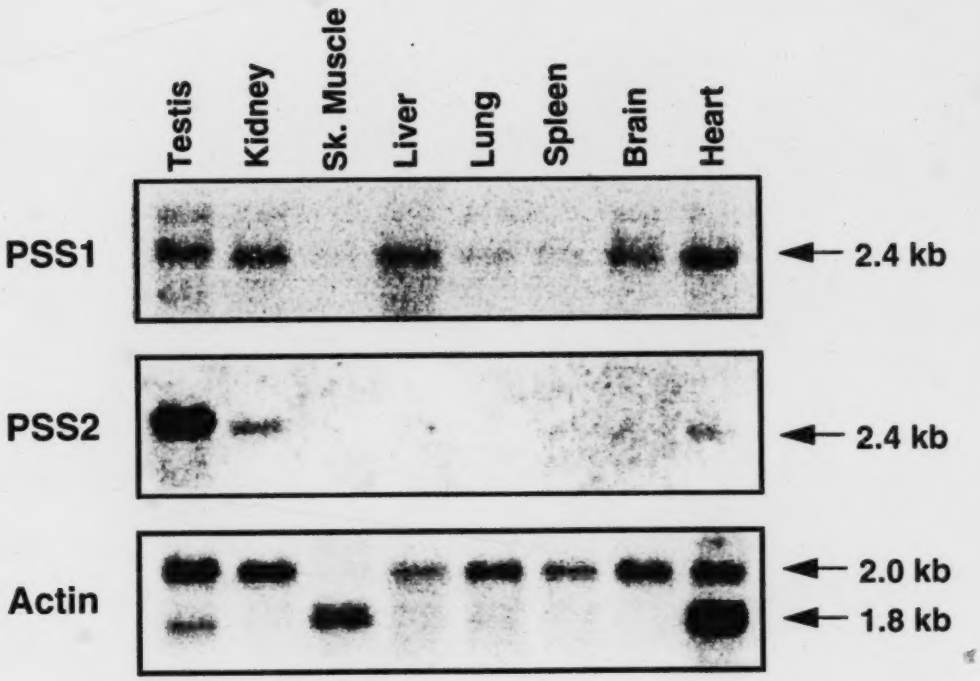
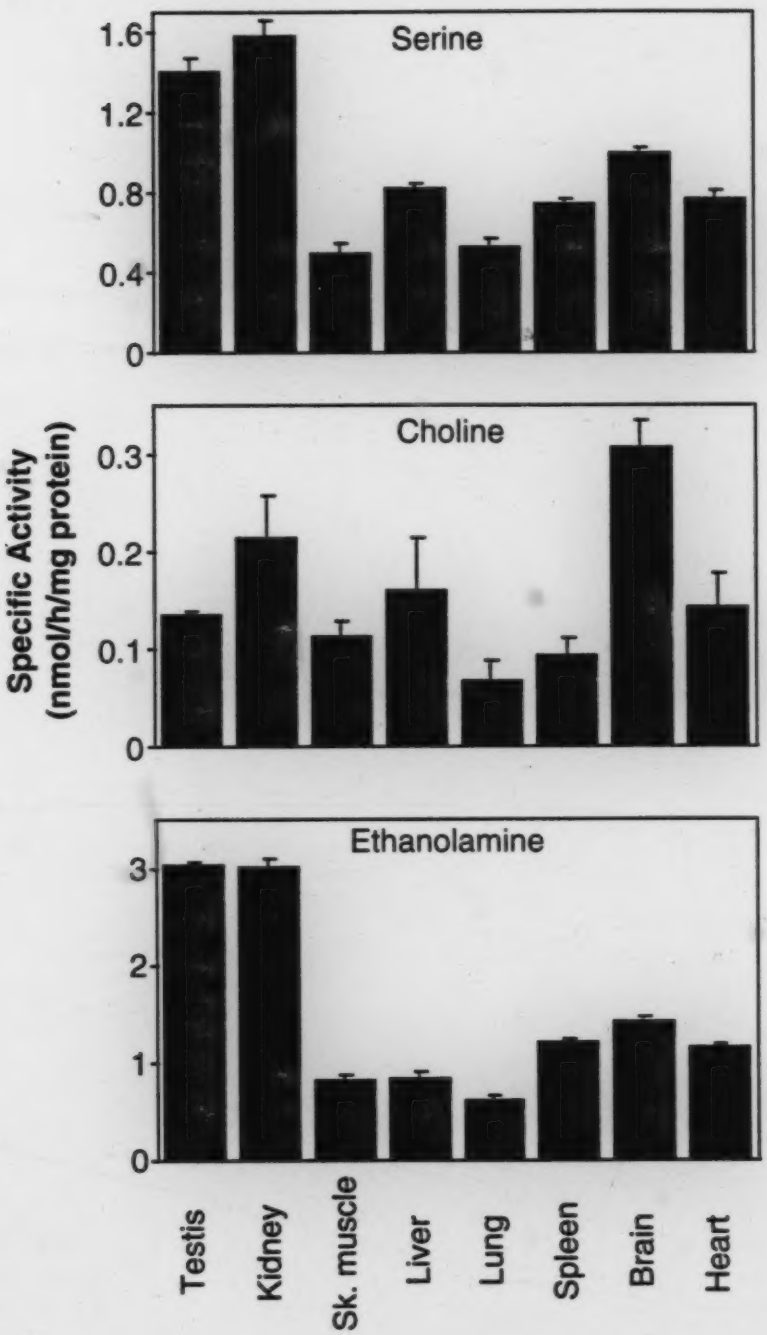


Fig. 3.40. Distribution of PSS1 and PSS2 gene expression in murine tissues.

A mouse multiple-tissue Northern blot containing approximately 2 μg of poly A^+ RNA was purchased from Clontech Laboratories and probed with mouse liver PSS1 and PSS2 cDNAs. The blot was also probed with β-actin cDNA as a loading control.

Fig. 3.41. PSS base-exchange activities of mouse tissues.

Base exchange activities were measured in tissue homogenates using [3-^{3}H]serine (Ser), [*methyl*-^{3}H]choline (Cho) and [1-^{3}H]ethanolamine (Etn). Data are averages ± S.D. of duplicate analyses from 2 independent experiments.


Serine
1.6
1.2
0.8
0.4
0
Choline
0.3
0.2
0.1
0
Specific Activity
(nmol/h/mg protein)
Ethanolamine
3
2
1
0
Testis
Kidney
Sk. muscle
Liver
Lung
Spleen
Brain
Heart

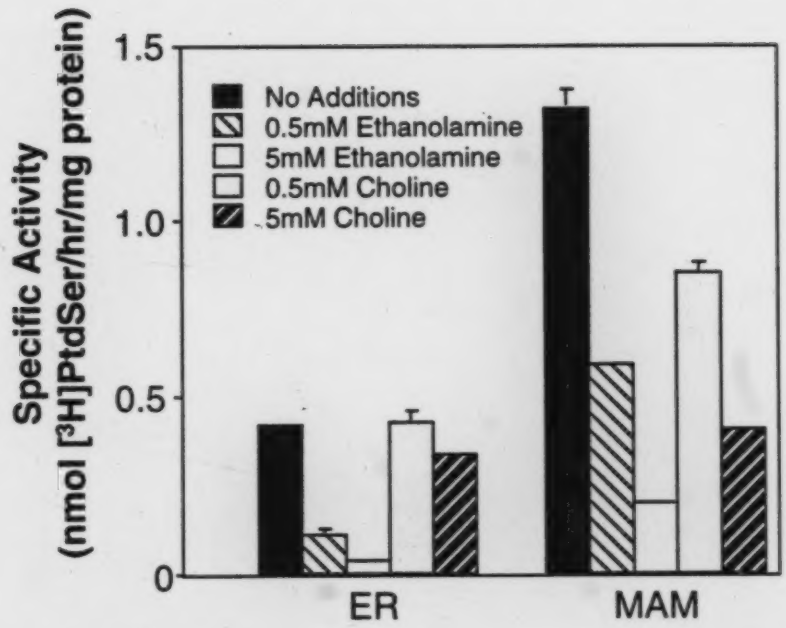


Fig. 3.42. PSS activity in MAM, but not ER, is inhibited by choline, while ethanolamine inhibits PSS activity in both MAM and ER.

PSS activity was measured in ER and MAM fractions that were isolated from murine liver in the presence and absence of 0.5 mM or 5 mM choline or ethanolamine. Data are averages ± S.D. of triplicate analyses from 2 independent experiments. Some error bars are too small to be visible.

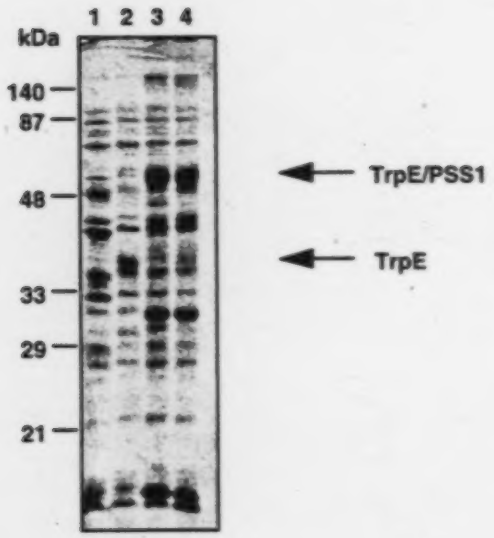


Fig. 3.43. Expression of TrpE/PSS1 fusion protein in *E.coli*.

Cells were grown for 2 h at 37 °C until A_{600} = 0.4. The culture was then diluted 10-fold into fresh medium and allowed to grow for 1 h, at which time cells were induced by the addition of 0.5 ml of 2 mg/ml indoleacrylic acid and allowed to grow for 4 h. The insoluble protein fractions were isolated from *E. coli* and *E. coli* expressing TrpE or TrpE/PSS1 and separated by 12% SDS-PAGE. Lane 1, *E.coli*; lane 2, TrpE; lane 3, TrpE/PSS1 uninduced; lane 4, TrpE/PSS1 induced.

CHAPTER 4

DISCUSSION AND CONCLUDING REMARKS

4.1 Cloning and expression of murine liver PSS1 and PSS2 in M.9.1.1 cells

As stated in Chapter 1, the primary objectives of this thesis were to isolate the cDNAs for PSS1 and PSS2 from murine liver, and by heterologous expression, examine their roles in phospholipid metabolism in two different cell culture model systems; the M.9.1.1 mutant and in McArdle RH7777 cells. Sequence analysis revealed that both the cDNA and predicted amino acid sequence for PSS1 and PSS2 from murine liver had very little similarity, and are likely encoded by different genes. As evidence that the isolated cDNAs encoded PSS1 and PSS2, respectively, the cDNAs were heterologously expressed in M.9.1.1 cells which are mutant CHO cells deficient in PSS1 activity [96]. When PSS1 cDNA was expressed, the ethanolamine auxotrophy of the M.9.1.1 cells was eliminated, the base exchange activities with serine, choline and ethanolamine were increased, and the levels of PtdSer and PtdEtn in these cells were restored to normal. These results are consistent with those of Kuge *et al.* (1991), where PSS1 from CHO-K1 cells was able to complement the PSA-3 mutant, and strongly suggests that the PSS1 cDNA isolated from murine liver encodes PSS1 [104].

Expression of PSS2 in M.9.1.1 cells resulted in an increase in serine and ethanolamine exchange activities only. No increase in choline exchange activity was observed, which is consistent with substrate preferences of PSS2. Unlike PSS1, PSS2 was only partially able to restore normal growth of M.9.1.1 cells, both in the presence and absence of ethanolamine in the culture medium. Expression of PSS2 was also able to increase PtdSer levels of M.9.1.1 cells. However, PtdEtn levels only returned to normal when ethanolamine was included in the culture medium. The lack of restoration of PtdEtn

levels might explain why the cells expressing only PSS2 did not grow normally when cultured without ethanolamine in the culture medium. It is possible that since PtdEtn is the only substrate for PSS2 in intact cells, PtdEtn is being depleted at a faster rate in M.9.1.1 cells expressing PSS2. For some reason, PtdEtn produced by decarboxylation of PtdSer does not appear to contribute an adequate source of PtdEtn for PSS2, since for every molecule of PtdEtn consumed by PSS2, PtdEtn can potentially be replaced by decarboxylation of PtdSer. This suggests that PtdEtn synthesized by the CDP-ethanolamine pathway serves as the primary source of PtdEtn for PSS2 in these cells or that insufficient PtdEtn is made from PtdSer by PSS2. This might explain why PtdEtn levels in M.9.1.1 cells expressing PSS2 are reduced when cultured in the absence of ethanolamine. PtdEtn from the CDP-ethanolamine is utilized by PSS2, and cannot be regenerated in the absence of ethanolamine, resulting in a decrease in PtdEtn levels. In M.9.1.1 cells expressing PSS1, PtdEtn levels returned to normal when cultured either in the presence or absence of ethanolamine, since PtdCho is the substrate for PSS1 and is abundant [218]. The inability of murine liver PSS2 to restore completely growth and PtdEtn levels in M.9.1.1 cells does not coincide with the findings of Kuge *et al.* (1997), who showed that PSS2 from CHO-K1 cells could complement the growth defect and restore normal PtdEtn levels of the PSA-3 mutant [114]. Since M.9.1.1 and PSA-3 cells are phenotypically similar, it is unlikely that these observations can be attributed to variations between these two cell lines.

4.2 Regulation of PtdSer synthesis by PSS1 and PSS2

In M.9.1.1 cells expressing murine PSS1 or PSS2, the serine-exchange activity was ~4-fold higher than that of control M.9.1.1 cells and ~2-fold higher than wildtype CHO-K1 cells when measured in cell lysates. The synthesis of PtdSer was also examined in intact M.9.1.1 cells expressing murine PSS1 or PSS2 by labeling these cells with [^{3}H]serine and measuring its incorporation into PtdSer. When PtdSer synthesis was measured, the amount of radiolabeled serine in PtdSer in cells expressing PSS2, although greater than that of M.9.1.1 cells, did not exceed that of CHO-K1 cells. However, cells expressing PSS1 incorporated ~3-fold and ~2-fold more radiolabel into PtdSer than M.9.1.1 cells and CHO-K1 cells, respectively. These findings do not correspond exactly to those of Kuge *et al.* (1998), who suggested that PtdSer synthesis by PSS1 and PSS2 are subject to regulation by end-product inhibition [117]. They found that addition of exogenous PtdSer inhibited PtdSer synthesis by directly decreasing the activity of PSS1 and PSS2. It was not demonstrated if these effects were due to modification of the PSS1 protein or by altered gene expression. The data obtained from experiments in which murine PSS1 and PSS2 were expressed in McArdle RH7777 cells and M.9.1.1 cells suggests that although PSS2 from murine liver appears to be subject to regulation by end-product inhibition, PSS1 does not. The reason for this is not yet clear. It has been proposed that there is a regulatory element present in both PSS1 (PNGPFT**R**^{95}PHPALWRM) and PSS2 (KDGPFS**R**^{97}PHPAYWRF) that contains an arginine residue responsible for this end-product inhibition mechanism. Mutation of Arg-95 of PSS1 to lysine in the CHO-K1 mutant 29 or by site-directed mutagenesis of PSS1,

demonstrated that this residue is indeed essential for the inhibition of PtdSer synthesis by PtdSer, in CHO-K1 cells [117]. It has not yet been determined if this key arginine residue also is involved in regulating PtdSer synthesis by CHO-K1 PSS2, however. This arginine residue that is proposed to be involved in the regulation of PtdSer synthesis is also present in murine PSS1 and PSS2. In addition, this putative regulatory site from both PSS1 and PSS2 from CHO-K1 cells exactly matches the corresponding regions of PSS1 and PSS2 from murine liver over a length of 112 and 208 amino acids, respectively. It is possible that these regions do impose some regulatory inhibition on PtdSer synthesis, but there must be additional elements required that are absent or repressed in murine liver PSS1. As mentioned in Section 3.1, murine liver PSS1 contains a tyrosine and serine at positions 424 and 425, respectively, that are absent in CHO-K1 PSS1. Therefore, these two amino acids, which are potential targets for phosphorylation, may play a role in regulating PtdSer biosynthesis. However, these two amino acids are not part of any known consensus sequences for protein phosphorylation. This does not preclude the possibility that Tyr^{424} and Ser^{425} are involved in regulating PtdSer synthesis in another manner. A possible future experiment would be to alter these two residues from murine PSS1 and determine if PSS1 would then become subject to end-production inhibition.

The effects of expression of murine and human PSS1 and murine PSS2 in McArdle RH7777 cells, which already have endogenous PSS activity, were also examined. Although both PSS1 and PSS2 were each expressed several-fold in McArdle RH7777 cells, very different effects on phospholipid metabolism were observed. Expression of murine and human PSS1 in McArdle 7777 cells resulted in increased *in vitro* base

exchange activity which was accompanied by a corresponding increase in the rate of incorporation of [^{3}H]serine into PtdSer and PtdEtn in intact cells. However, in Mc/PSS2 cells, although the *in vitro* activity of PSS2 was increased, there was no increase in PtdSer or PtdEtn synthesis. This correlates well with the results observed in M.9.1.1 cells expressing murine PSS1 and PSS2, again suggesting that PSS1 and PSS2 from murine liver are differentially regulated. PSS2 is regulated by end-product inhibition whereas PSS1, from mouse or human, is not.

Studies in McArdle RH7777 cells imply that the metabolism of PtdSer and PtdEtn is coordinately regulated so that cellular levels of these lipids remain constant. No changes in phospholipid levels were observed in either Mc/PSS1 or Mc/PSS2 cells, even though Mc/PSS1 cells, at least, are producing more PtdSer and PtdEtn, compared to control McArdle RH7777 cells. Since PtdSer synthesis is increased in Mc/PSS1 cells compared to control McArdle RH7777 cells, PSS1 from murine liver appears not to be regulated by end-product inhibition. Although the synthesis of PtdSer and PtdEtn was increased in Mc/PSS1 cells, their PtdSer and PtdEtn content was not altered, suggesting that an alternative mechanism must exist to maintain constant phospholipid levels. When PSS1 activity was expressed in McArdle RH7777 cells, homeostasis of PtdSer and PtdEtn was maintained by increased conversion of PtdSer to PtdEtn by decarboxylation and by increased degradation of PtdSer and/or PtdEtn derived from PtdSer. These findings are similar to those from a study in which CTP:phosphocholine cytidylyltransferase, the rate-limiting enzyme of PtdCho biosynthesis via the CDP-choline pathway, was expressed in COS cells [219]. Despite a greatly increased rate of

PtdCho synthesis in these transfected cells, PtdCho levels barely increased. However, the rate of PtdCho degradation was enhanced, presumably as a mechanism for maintaining a constant level of PtdCho. Similarly, when PSS from *E. coli* was overexpressed in *E. coli*, a 100-fold increase in PSS activity was observed in bacterial lysates, but there was no alteration of the phospholipid composition of these cells [220].

The increased conversion of PtdSer to PtdEtn in cells expressing PSS1 is most likely the direct result of increased PtdSer synthesis. Production of PtdSer-derived PtdEtn appears not to be limited by the activity of the decarboxylase but rather by availability of the substrate, PtdSer [152]. PtdSer supply is, in turn, regulated by either the rate of PtdSer synthesis or the rate of translocation of PtdSer from its site of synthesis on the ER to the site of the decarboxylase on the outer aspect of inner mitochondrial membranes [60]. Hence, an increased supply of PtdSer to mitochondria would be expected to be translated into an increased production of PtdEtn by the decarboxylase.

A second consequence of expression of PSS1 was that degradation of PtdSer and possibly PtdSer-derived PtdEtn was increased, although the degradation of PtdEtn derived from the CDP-ethanolamine pathway was not increased. Most likely, the enhanced degradation was of PtdSer rather than of PtdEtn since in primary hepatocytes newly-made PtdSer is rapidly degraded whereas PtdSer-derived PtdEtn is not significantly degraded [138].

4.3 Post-transcriptional regulation of murine PSS1 and PSS2

Northern blot analysis of PSS1 and PSS2 mRNA levels in M.9.1.1 cells expressing PSS1 and PSS2 revealed that these two transcripts were expressed at very high levels (~14-fold and ~300-fold, respectively) compared to control M.9.1.1 cells and CHO-K1 cells. However, this high level of expression of PSS1 and PSS2 was accompanied by only moderate increases in PSS1 and PSS2 activity (~4-fold). These data suggest that PSS1 and, especially, PSS2 can be subject to post-transcriptional regulation, in a negative manner. Since no antibodies are available for either murine PSS1 or PSS2, it is not known whether these two enzymes can be regulated at the level of translation or post-translationally. Yeast PSS activity has been shown to be regulated by the level of PSS mRNA abundance [221]. However, since the reactions catalyzed by the yeast and mammalian enzymes are completely different, no clear comparison can be made.

4.4 Tissue distribution and subcellular localization of PSS1 and PSS2 in murine tissues

In Section 3.12, the distribution of PSS1 and PSS2 gene expression was examined in eight different murine tissues by Northern blot analysis. PSS1 mRNA was abundant in brain, heart, liver, kidney and testis, with low levels of expression in skeletal muscle, lung and spleen. In contrast, PSS2 mRNA was found predominantly in heart and kidney, and was most abundant in testis. These data, along with data obtained by assaying the serine, choline and ethanolamine exchange activities of these tissues, seem to suggest that the brain possesses mostly PSS1 activity, while kidney and testis possess mostly PSS2

activity. In the other tissues examined, it was difficult to distinguish between the contribution of PSS1 and PSS2. These tissues probably contain mostly PSS1, since PSS2 mRNA was difficult to detect in these tissues by Northern blot analysis. The reason why the tissue distribution of PSS1 and PSS2 differs is not presently understood. It is possible that each enzyme produces different pools of PtdSer that have specific cellular functions. For example, in the brain PSS1 gene expression appears to be greater than that of PSS2, and may function to produce PtdSer enriched in 22:6, since PtdSer in the brain has a higher proportion of 22:6 than most other tissues. The generation of PSS1 and PSS2 knockout mice would facilitate the understanding of the roles these two enzymes play in lipid metabolism in these tissues. If the genes for PSS1 or PSS2 were disrupted, the contribution of PtdSer produced by each of these enzymes to the blood coagulation process, cell signaling and the removal of apoptotic cells could be examined in great detail.

In addition to the apparently different tissue distribution of PSS1 and PSS2, these two proteins also could potentially reside in different subcellular compartments. The data from competition experiments in Section 3.12 suggests that PSS1 resides in MAM, while PSS2 resides in ER and possibly MAM, which could imply that if MAM is involved in the transport of PtdSer to the mitochondria that PSS1 might produce most of the PtdSer that is decarboxylated to PtdEtn. In contrast, Saito *et al.* (1996) have suggested that PSS1 is localized to both microsomes and MAM [105]. Production of specific antibodies against both PSS1 and PSS2 for immunoblot analysis of liver membrane fractions will help determine the exact subcellular localization of these two enzymes.

4.5 Coordinate regulation of PtdEtn synthesis

As mentioned in Section 4.2, PtdEtn production from decarboxylation of PtdSer was increased in Mc/PSS1 cells, compared to that in control McArdle RH7777 cells. However, there was no increase in PtdEtn mass in cells expressing PSS1. In order to maintain PtdEtn at a constant level in these cells, it was determined that the increased synthesis of PtdSer-derived PtdEtn from the expression of PSS1 in McArdle RH7777 cells resulted in the inhibition of synthesis of PtdEtn via the CDP-ethanolamine pathway (Fig. 3.13). This conclusion was deduced from the greatly reduced incorporation of [^{3}H]ethanolamine into PtdEtn which could not be accounted for by an increased degradation of ethanolamine-derived PtdEtn. Moreover, the amounts of [^{3}H]ethanolamine-labeled precursors of PtdEtn were increased indicating that the CDP-ethanolamine pathway for PtdEtn synthesis was inhibited in Mc/PSS1 cells compared to control cells. A similar phenomenon was observed in the CHO-K1 mutant 29 cell line, which had unregulated, elevated PtdSer synthesis which was accompanied by a corresponding decrease in the synthesis of PtdEtn by the CDP-ethanolamine pathway [118]. However, this inhibition of the CDP-ethanolamine pathway was observed in M.9.1.1 cells expressing murine PSS2, but was not observed in McArdle RH7777 cells expressing murine PSS2, which showed no increase in PtdSer synthesis, suggesting that the CDP-ethanolamine pathway is inhibited only when the rate of synthesis of PtdSer and/or PtdEtn in the cell is increased. Therefore, the step at which the CDP-ethanolamine pathway was modified when PSS1 was expressed was examined. Under most metabolic conditions the rate-limiting enzyme of this pathway is considered to be ET [169, 170,

176]. However, the total *in vitro* activity of ET was unaffected by expression of PSS1. The distribution of ET between a small active pool and a large inactive pool may have been altered, and such a change would not have been detected in our experiments. Unlike the cytidylyltransferase of the CDP-choline biosynthetic pathway [180], there is no evidence that the activity of ET is regulated by reversible translocation to and from membranes [176]. ET is presumed to be a cytosolic protein that neither requires lipids for activity nor is tightly associated with membranes [173, 177, 178, 189]. However, some association of ET with ER membranes has been detected in immunogold electron microscopy studies [173]. The cDNA sequences of ET [175] and CTP:phosphocholine cytidylyltransferase [179] share some similarities, especially at the N-termini of the corresponding proteins. CTP:phosphocholine cytidylyltransferase contains an amphipathic alpha helical C-terminal domain that has been proposed to mediate membrane association [180]. From analysis of the cDNA sequence of ET [175] it appears that ET might also have an amphipathic alpha-helical domain close to the C-terminus that could potentially form a loose association with membranes.

The supply of DAG has also been implicated in the regulation of the CDP-ethanolamine pathway [189, 216] by virtue of this lipid being a substrate for ethanolaminephosphotransferase. Similarly, under some metabolic conditions, DAG can regulate the CDP-choline pathway for PtdCho synthesis at the level of the cholinephosphotransferase [186, 217, 222]. However, no difference was detected in the DAG content of control cells and Mc/PSS1 cells although these experiments would not have detected a change in a small localized pool of this lipid. The DAG content of total

cellular membranes also appeared to be unchanged. However, in cells expressing PSS1, the cellular content of TAG was 40% less than in control cells. For each molecule of PtdSer synthesized, one molecule of phospholipid, and hence one molecule of DAG, is consumed. If the reservoir of stored TAG in the cell served as a precursor pool of DAG, a reduction in TAG mass might result from the increased demand for DAG in the cells expressing PSS1. Therefore, expression of PSS1 might deplete a specific pool of DAG that is used for PtdEtn or PtdCho synthesis via EPT.

Since it appears that increasing the synthesis of PtdEtn from the decarboxylation of PtdSer results in the inhibition of the CDP-ethanolamine pathway, it was of interest to determine if over-production of PtdEtn via the CDP-ethanolamine pathway, by expression of ET, inhibits the synthesis and/or decarboxylation of PtdSer. Voelker and Frazier (1986) demonstrated that although M.9.1.1 cells have reduced PtdSer synthesis, and consequently reduced PtdEtn synthesis from PtdSer, that there is an increase in synthesis of PtdEtn via the CDP-ethanolamine pathway, presumably as a compensatory mechanism [96]. Expression of human ET in McArdle RH7777 cells resulted in an increase in *in vitro* ET activity, however, there was no increase in the incorporation of [^{3}H]ethanolamine into CDP-ethanolamine or PtdEtn in intact cells. It has now become apparent that although this cDNA encodes the entire coding region for ET, it lacks an 18 amino acid sequence present in rat liver ET between residues 179 and 180 (Fig. 3.36) that may be required for proper functionality in mammalian cells which might account for the lack of CDP-ethanolamine synthesis. It is currently unclear why when the cDNA encoding human ET is expressed in yeast that it is able to increase the synthesis of both

CDP-ethanolamine and PtdEtn [175]. Since PtdEtn synthesis by the CDP-ethanolamine pathway was not increased by expressing human ET cDNA in McArdle RH7777 cells, the effects on the production of PtdEtn from the decarboxylation of PtdSer could not be examined in detail.

As previously mentioned, a cDNA encoding ET has also been isolated from rat liver which appears to be full-length (Dr. M. Houweling, unpublished results). Expression of this cDNA in McArdle RH7777 cells resulted in a 3-fold increase in *in vitro* ET activity compared to control McArdle RH7777 cells, and unlike human ET, also resulted in a 15-fold increase in the amount of [^{3}H]ethanolamine incorporated into CDP-ethanolamine, in intact cells. However, when the incorporation of [^{3}H]ethanolamine into PtdEtn was examined, it was instead reduced by 44%. It is possible that an increased synthesis of PtdEtn by the CDP-ethanolamine pathway is accompanied by an increase in the degradation of PtdEtn as well, in order to maintain a constant level of PtdEtn within a cell. Consequently, the rate of degradation of PtdEtn could exceed the rate of synthesis, resulting in lower levels or radiolabeled PtdEtn. Analysis of the intermediates of the CDP-ethanolamine pathway, however, revealed that there was no accumulation of glycerophosphoethanolamine, phosphoethanolamine or ethanolamine, indicating that there was no increase in degradation of PtdEtn in McArdle RH7777 cells expressing rat liver ET. Transient expression of rat liver ET cDNA in COS cells also showed no stimulation of PtdEtn synthesis (Dr. M. Houweling, personal communication). This result was surprising, but suggested that CDP-ethanolamine itself might be capable of regulating the synthesis of PtdEtn, possibly by inhibiting the activity of EPT. If EPT is

indeed regulated by CDP-ethanolamine, these observations could provide evidence that EPT may have a more important role in regulating PtdEtn synthesis than previously thought. It has generally been accepted that only the supply of DAG, and not CDP-ethanolamine, regulates the third step of the CDP-ethanolamine pathway. Since PtdEtn synthesis by the CDP-ethanolamine pathway was not increased, it could not be determined if increased PtdEtn synthesis from this pathway can decrease the synthesis of PtdEtn from PtdSer. Possibly, expression of EPT might be able to stimulate PtdEtn synthesis, although no mammalian cDNAs encoding this enzyme are available to date.

Although no effect on the CDP-ethanolamine pathway was observed in McArdle RH7777 cells expressing human ET, we did observe a small, but significant, decrease in the incorporation of [^{3}H]serine into PtdSer, but not PtdEtn derived from PtdSer. This ruled out the possibility that increased expression of human ET could inhibit PtdEtn synthesis from PtdSer. However, we did not observe the same result in McArdle RH7777 cells expressing rat liver ET. Instead, there was no difference in the amount of radiolabeled serine incorporated into PtdSer or PtdEtn derived from PtdSer in McArdle RH7777 cells expressing rat ET compared to control McArdle RH7777 cells. Currently, we not do have any explanation for these results.

4.6 Concluding remarks

The presence of two genetically distinct PtdSer synthases poses interesting questions to lipidologists: What are the physiological roles of these two enzymes and are they both required for a cell to function normally? Although it has clearly been shown

that in cells in culture, either PSS1 or PSS2 is sufficient for cell growth, it has not been determined if other cellular functions, such as PKC activation, are effected. Additionally, the differential expression of PSS1 and PSS2 in several murine tissues seems to suggest that these two enzymes are not interchangeable and each serves a specific purpose. The production of PSS1 and PSS2 knockout mice would provide valuable models to study the roles of these two enzyme an intact animals. The effects on the blood coagulation process and removal of apoptotic cells could be evaluated to determined if PtdSer generated by PSS1 and/or PSS1 is preferentially utilized for these processes.

Although the biosynthetic pathway for PtdSer in animal cells has been well established, the mechanisms by which this process is regulated is poorly understood. Only recently has it been determined that PtdSer biosynthesis in CHO-K1 cells can be regulated by a feedback inhibition mechanism [117, 118]. However, we have described a somewhat different mechanism involved in regulating PtdSer synthesis by murine PSS1 and PSS2. An increased synthesis of PtdSer, induced by expression of murine liver PSS1 in rat hepatoma cells, but not PSS2 cDNA, increases the production of PtdEtn from the PtdSer decarboxylation pathway and inhibits the synthesis of PtdEtn via the CDP-ethanolamine pathway. It appears that PtdSer synthesis by murine PSS1 is regulated by increased turnover/degradation of PtdSer and PtdEtn, while PSS2 can be regulated by feedback inhibition. These observations suggest that the synthesis and decarboxylation of PtdSer, as well as the synthesis of PtdEtn from the CDP-ethanolamine pathway and the degradation of PtdSer and/or PtdEtn, are coordinately regulated so that constant cellular levels of these phospholipids are maintained.

The reciprocal regulation of PtdEtn synthesis from the decarboxylation of PtdSer and via the CDP-ethanolamine pathway could not be demonstrated. Although increasing the activity of ET, the rate-limiting enzyme of the CDP-ethanolamine pathway, by expression of the cDNA encoding rat liver ET, stimulated the synthesis of CDP-ethanolamine, this did not translate into an increase in PtdEtn synthesis. Surprisingly, the synthesis of PtdEtn actually appeared to be inhibited. This suggested that even though the conversion of CDP-ethanolamine to PtdEtn by EPT can be regulated by the supply of DAG, this reaction might also be regulated by the supply of CDP-ethanolamine.

CHAPTER 5

APPENDIX 1 - SUPPLEMENTARY FIGURES AND DATA

5. Appendix 1 - Supplementary figures and data

This appendix contains additional data that corresponds to the following figures in which one representative experiment was shown in Chapter 3 (results section):

Fig 3.5	Fig. 5.1. Base-exchange enzyme activities of M.9.1.1 cells and control M.9.1.1 cells expressing mouse liver PSS1
Fig. 3.11	Fig. 5.2. The rate of turnover/degradation of [^{3}H]serine-derived PtdSer and PtdEtn in control McArdle RH7777 cells and Mc/PSS1 cells is increased
Fig. 3.12	Fig. 5.3. [^{3}H]ethanolamine-derived metabolites of the CDP-ethanolamine pathway accumulate in Mc/PSS1 cells
Fig. 3.14	Fig. 5.4. Rate of degradation of [^{3}H]ethanolamine-derived PtdEtn is not increased in Mc/PSS1 cells
Table I	Fig. 5.5. ET activity is not reduced in Mc/PSS1 cells
Fig. 3.20	Fig. 5.6. Expression of murine PSS2 cDNA in M.9.1.1 cells complements the growth defect of this cell line
Fig. 3.23	Fig. 5.7. The turnover of [^{3}H]ethanolamine-derived PtdEtn in M.9.1.1 cells expressing murine PSS2 is not altered
Fig. 3.24	Fig. 5.8. Metabolism of [^{3}H]serine-derived PtdSer and PtdEtn in control M.9.1.1 cells and M.9.1.1 cells transfected with murine PSS2
Fig. 3.35	Fig. 5.9. Incorporation of [^{3}H]serine into PtdSer and PtdEtn of McArdle RH7777 cells and McArdle RH7777 cells expressing human ET
Fig. 3.38	Fig. 5.10. McArdle RH7777 cells expressing rat liver ET have increased content of [^{3}H]CDP-ethanolamine, but not PtdEtn, compared to control McArdle RH7777 cells

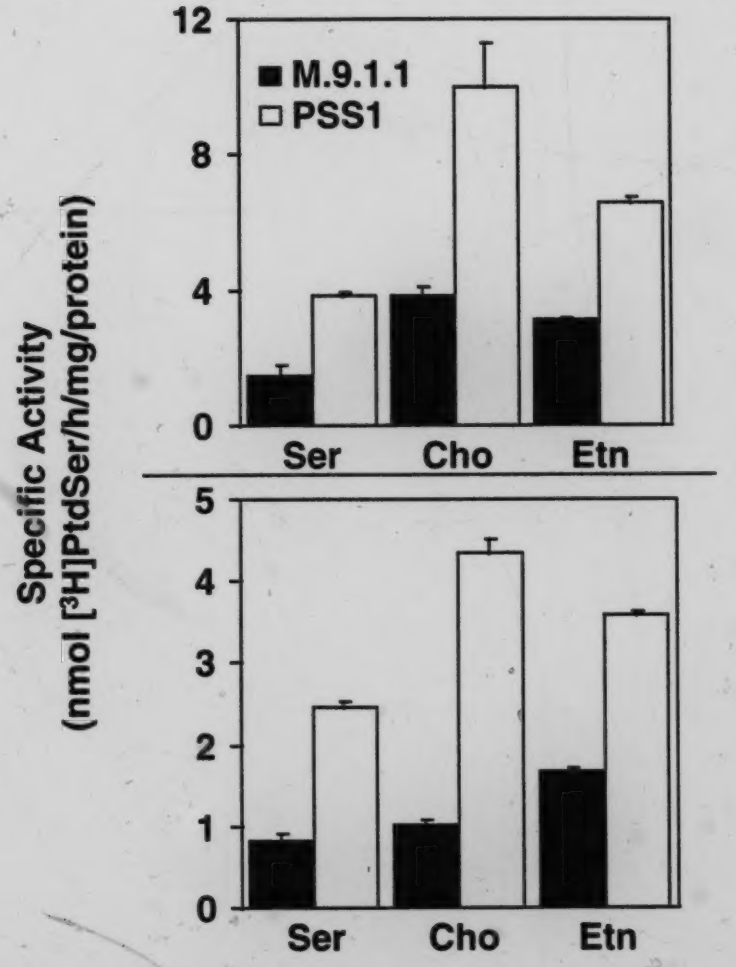


Fig. 5.1. Base-exchange enzyme activities of M.9.1.1 cells and control M.9.1.1 cells expressing mouse liver PSS1.

Base exchange activities were measured using [3-^{3}H]serine (Ser), [1-^{3}H]ethanolamine (Etn) and [*methyl*-^{3}H]choline (Cho) in cellular lysates from control M.9.1.1 cells transfected with empty vector (solid bars) and M.9.1.1 cells expressing PSS1 cDNA (open bars). Data are averages ± S.D. of triplicate analyses.

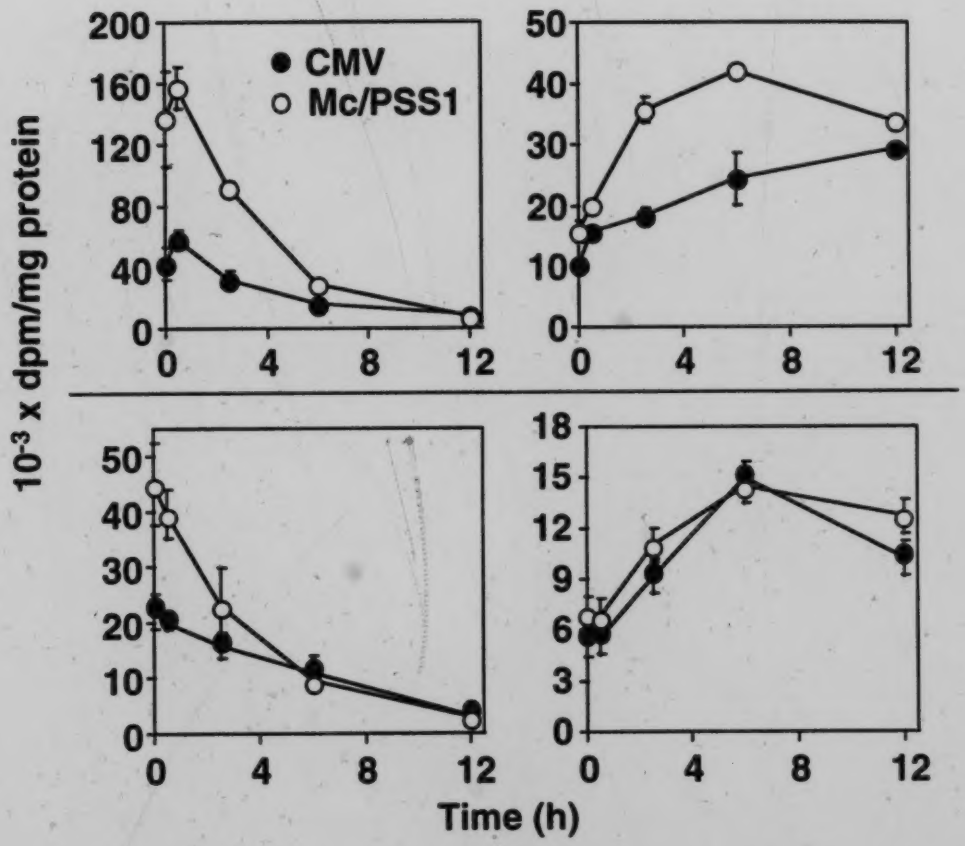
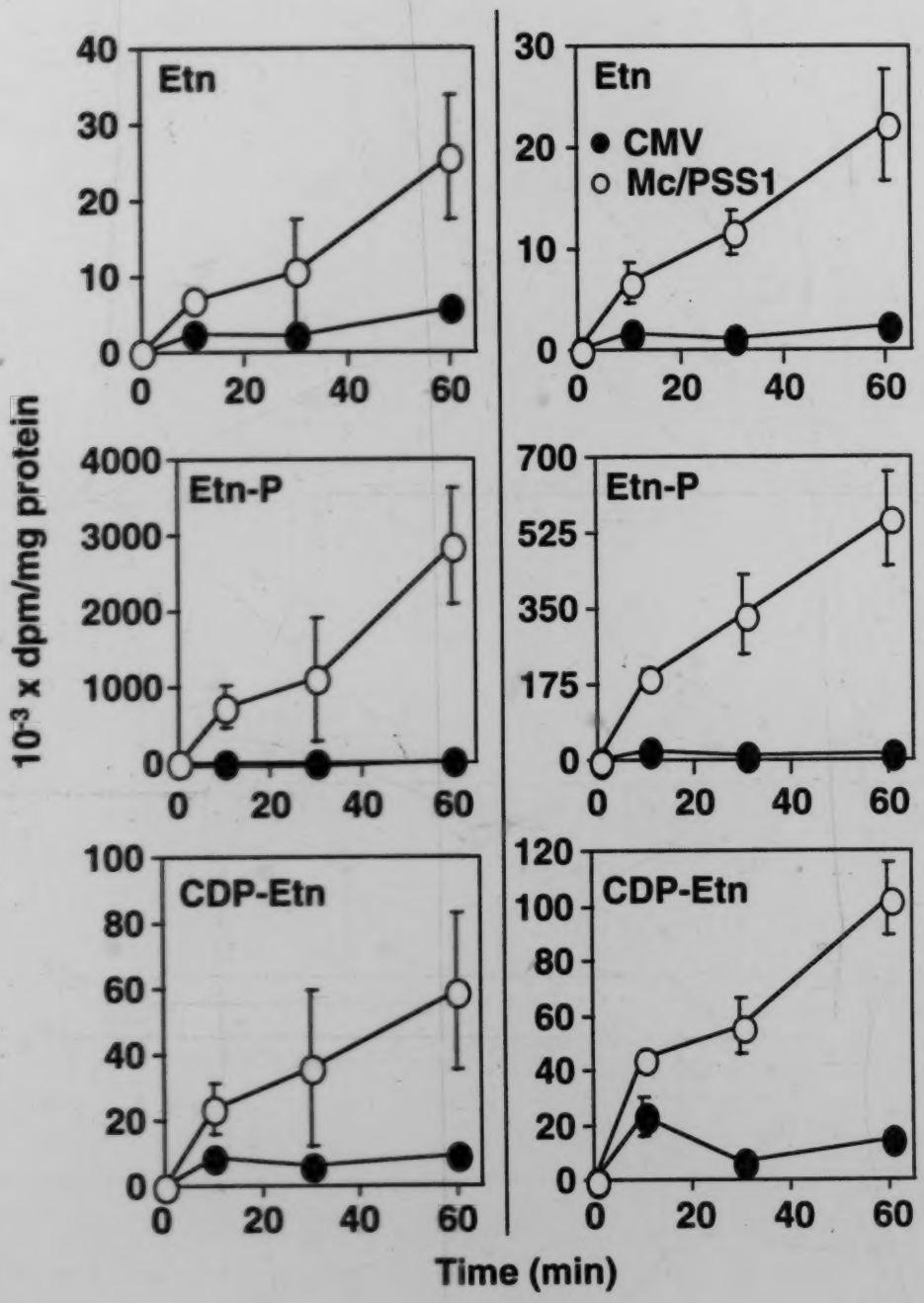


Fig. 5.2. The rate of turnover/degradation of [^{3}H]serine-derived PtdSer and PtdEtn in control McArdle RH7777 cells and Mc/PSS1 cells is increased.

Cells were incubated for 1 h in medium containing [^{3}H]serine (20 μCi/dish). The medium was then removed and fresh medium containing 1 mM unlabeled serine was added. At the indicated times cells were harvested and PtdSer (upper panel) and PtdEtn (lower panel) were extracted and separated by thin-layer chromatography. Control McArdle RH7777 cells (CMV, solid symbols); Mc/PSS1 cells (open symbols). Data are averages ± S.D. from triplicate analyses. Some error bars are too small to be visible.

Fig. 5.3. [^{3}H]ethanolamine-derived metabolites of the CDP-ethanolamine pathway accumulate in Mc/PSS1 cells.

Cells were incubated in medium containing [^{3}H]ethanolamine (1.5 μCi/dish) for the indicated times and the amount of radioactivity incorporated into the water-soluble intermediates of the CDP-ethanolamine pathway (Etn = ethanolamine, Etn-P = phosphoethanolamine, CDP-Etn = CDP-ethanolamine) was determined. Control McArdle RH7777 cells (CMV, solid symbols); Mc/PSS1 cells (open symbols). Data are averages ± S.D. of triplicate analyses. Some error bars are obscured by symbols.


Etn
Etn-P
CDP-Etn
CMV
Mc/PSS1
10^{-3} x dpm/mg protein
Time (min)

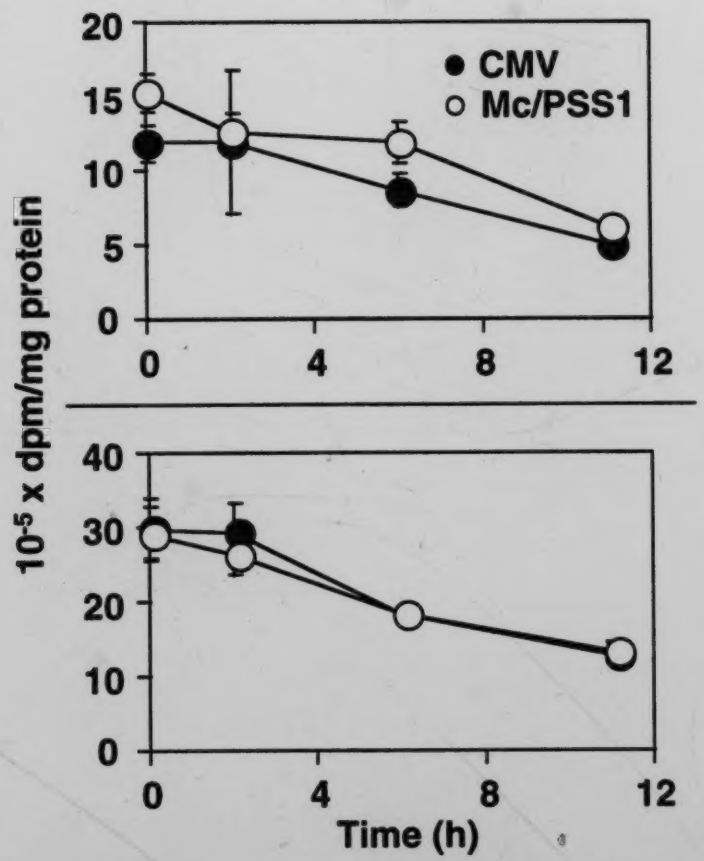


Fig. 5.4. Rate of degradation of [^{3}H]ethanolamine-derived PtdEtn is not increased in Mc/PSS1 cells.

Control McArdle RH7777 cells (CMV, closed symbols) and Mc/PSS1 cells (open symbols) were labeled with [^{3}H]ethanolamine (1.5 μCi/dish) for 24 h. The radiolabeled medium was removed and replaced with medium containing (2 mM) unlabeled ethanolamine. Cells were harvested at the indicated times and incorporation of radiolabel into PtdEtn was determined. Data are averages ± S.D. of triplicate analyses.

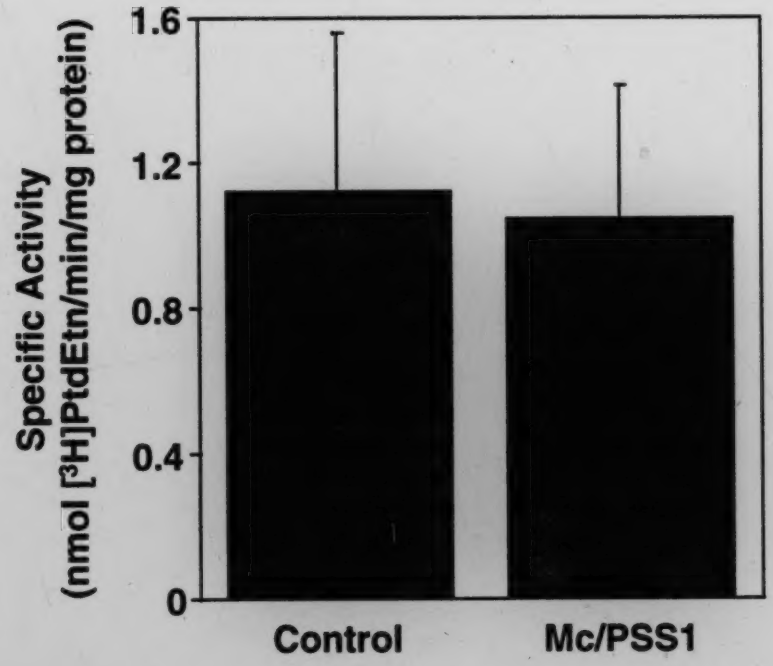


Fig. 5.5. ET activity is not reduced in Mc/PSS1 cells.

Cytosol was prepared from control McArdle RH7777 cells and Mc/PSS1 cells by centrifugation of cell lysates at 400,000 x *g* for 30 minutes and was assayed for ET activity. Data are averages ± S.D. of triplicate analyses.

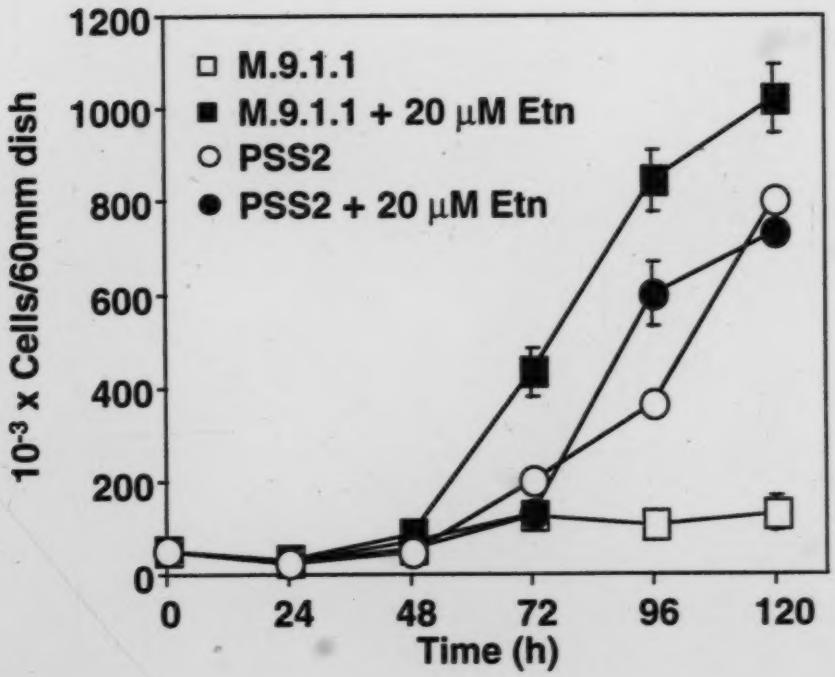


Fig. 5.6. Expression of murine PSS2 cDNA in M.9.1.1 cells complements the growth defect of this cell line.

Control M.9.1.1 cells (squares) and M.9.1.1 cells transfected with murine PSS2 cDNA (circles) were plated at a density of 5 x 10^4 cells/60 mm dish and cultured in Ham's F-12 medium supplemented with 10% delipidated fetal bovine serum with (solid symbols) or without (open symbols) 20 μM ethanolamine. Cells were harvested at 24 h intervals by trypsinization and counted. Data are averages ± S.D. of triplicate analyses. Some error bars are hidden by the symbols.

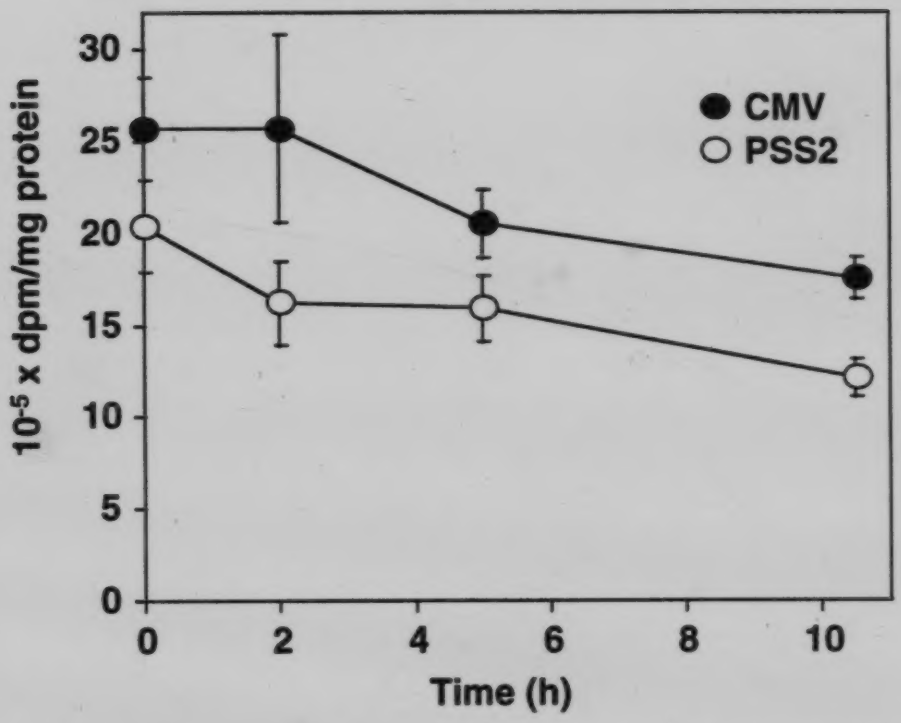


Fig. 5.7. The turnover of [^{3}H]ethanolamine-derived PtdEtn in M.9.1.1 cells expressing murine PSS2 is not altered.

Cells were incubated in medium containing [^{3}H]ethanolamine (1.5 μCi/dish) for 6 h after which the medium was removed and replaced with fresh medium containing 2 mM unlabled ethanolamine. Cells were then harvested and phospholipids were isolated. Solid symbols, control cells; open symbols, M.9.1.1 cells expressing PSS2. Data are averages ± S.D. of triplicate analyses.

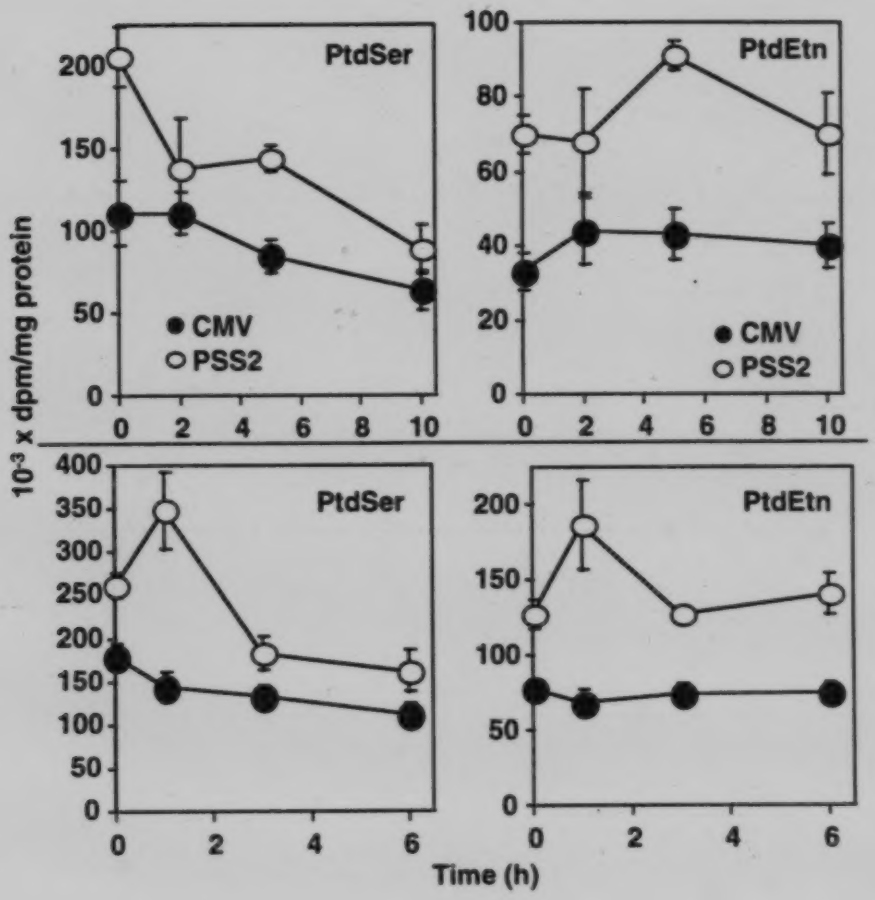


Fig. 5.8. Metabolism of [^{3}H]serine-derived PtdSer and PtdEtn in control M.9.1.1 cells and M.9.1.1 cells transfected with murine PSS2.

Cells were incubated for 6 h in medium containing [3-^{3}H]serine (15 μCi/dish) after which the medium was removed and replaced with fresh medium containing 2 mM unlabled serine. At the indicated times cells were harvested and PtdSer (panel A) and PtdEtn (Panel B) were isolated. Closed symbols, control M.9.1.1 cells; open symbols, M.9.1.1 cells expressing PSS2. Data are averages ± S.D. of triplicate analyses from one experiment.

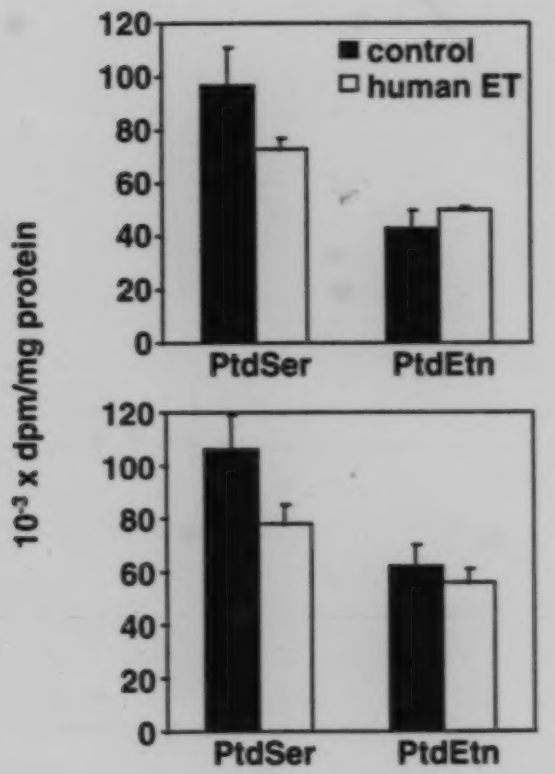


Fig. 5.9. Incorporation of [^{3}H]serine into PtdSer and PtdEtn of McArdle RH7777 cells and McArdle RH7777 cells expressing human ET.

Control RH7777 McArdle cells and McArdle RH7777 cells expressing human ET were incubated in medium containing [3-^{3}H]serine (20 μCi/dish) for 6 h. Cells were harvested and PtdSer and PtdEtn were extracted and separated by thin-layer chromatographyand the radioactivity in each lipid determined. Data are averages ± S.D. of triplicate analyses.

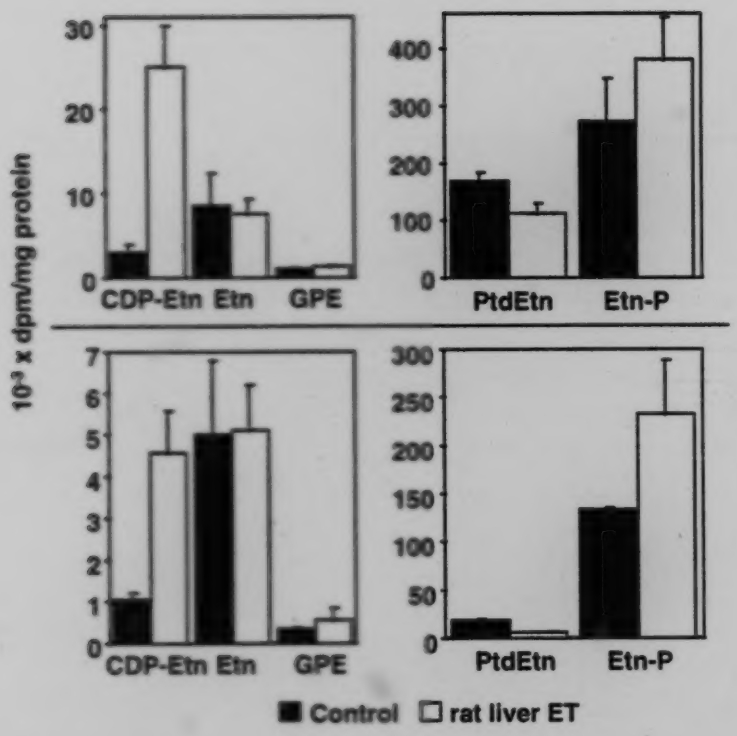


Fig. 5.10. McArdle RH7777 cells expressing rat liver ET have increased content of [^{3}H]CDP-ethanolamine, but not PtdEtn, compared to control McArdle RH7777 cells.

Cells were incubated in medium containing [^{3}H]ethanolamine (3 μCi/dish). After 4 h, cells were harvested and the amount of radioactivity in PtdEtn and the water soluble intermediates of the CDP-ethanolamine pathway, as well as glycerophosphoethanolamine, was determined. Ethanolamine, Etn; phosphoethanolamine, Etn-P, CDP-ethanolamine, CDP-Etn; glycerophosphoethanolamine, GPE. Data are averages ± S.D. of triplicate analyses.

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